Biochemical characterization of the *exuperantia* protein in *Drosophila*

by

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Abstract

Determination of the anteroposterior axis of a *Drosophila* embryo depends on the asymmetric localization of two maternal morphogens, *bicoid* (*bcd*) and *nanos* (*nos*), to the opposing poles of the egg. The RNAs of *bcd* and *nos* are localized at the anterior end and the posterior end of the egg, respectively, and are translated into two morphogen gradients which initiate and define the cell fates along the anteroposterior axis of the embryo. The localization of *bcd* mRNA begins at oogenesis and depends on several factors. The maternal gene *exuperantia* (*exu*) encodes a protein which is absolutely required for the localization of *bcd* mRNA. By molecular genetic analysis, the functional significance of several regions of the coding sequence of exu has been analyzed. A region that comprises 62 amino acid residues near the C-terminus was found to be dispensable for function. On the contrary, several regions were potentially important, as removal of these regions resulted in loss of the localization. Deletions of the regions affected different stages of the localization, suggesting the possibility of multiple functional domains directing different steps of the localization. Besides, exu protein displayed a spatial and temporal localization and the localization at the oocytes of early egg chambers seemed to be critical for localization of *bcd* mRNA.

Microtubules are required for the localization of exu protein. Localization of exu protein was disrupted in the presence of microtubule-destabilizing drugs, strongly suggesting interactions between exu protein and microtubules. Their interactions were confirmed by cosedimentation experiments. Exu protein was found to sediment in the presence of microtubules and thus can be classified, by definition, as a microtubule associated protein (MAP). Nonetheless, the interaction was specific to only one of the isoforms of exu protein - one of the hypo-phosphorylated isoforms was cosedimented with microtubules. Results led to a proposed function of exu protein which acts as a MAP and its functions might involve in the regulation of microtubule stability.

Abbreviations

Ag-Ab	Antigen-antibody
AP	Alkaline phosphatase
AP buffer	Alkaline phosphatase assay buffer
ATP	Adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate disodium
bp	base pair
BSA	Bovine serum albumin
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DIG	Digoxigenin
DOC	Deoxycholic acid
DTT	Dithiothreitol
dNTP	Deoxyribonucleoside triphosphates
dUTP	Deoxyuridine-triphosphate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FITC	Fluorescein isothiocyanate
g	gravity
GMM	Canada balsam mounting medium
GTP	Guanosine 5'-triphosphate
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
kD	kilo Dalton
MAPs	Microtubule associated proteins
MAP2	Microtubule associated protein 2
mg	milligram
ml	milliliter
MT	Microtubules
MTOC	Microtubule organizing center
NBT	Nitro blue tetrazolium
NP40	Tergitol NP-40 (Nonylphenoxy polyethoxy ethanol)
nt	nucleotide

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMSF	Phenylmethyl-sulfonyl fluoride
PTW	PBS with Tween 20
r.p.m.	revolutions per minute
SB	Sample buffer
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
Tween 20	Polyoxyethylene-sorbitan monolaurate
μg	microgram
μl	microliter
UTR	Untranslated region
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate

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CHAPTER 1

General Introduction

1.1 The formation of body axes

Development of an egg into an elaborate multi-cellular organism is a universal challenge to most living organisms. It involves cell division as well as accurate and precise determination of different cell fates within the embryo. Although different classes of animals display a wide range of diversities in their body forms, sizes and morphologies, they all share one common feature - they all have two fundamental body axes, the anteroposterior and the dorsoventral axes (Figure 1.1). Nonetheless, with the exception of *Drosophila,* the mechanism of body axes formation remains unclear in most of the organisms. In most dipterans, including *Drosophila*, the first step for development is establishment of body axes which allow subsequent developmental events to take place.

In *Drosophila*, the two body axes are established by asymmetric localization of signals in the embryo (Figure 1.2). The anterior determinant, *bicoid* (*bcd*) mRNA, and the posterior determinant, *nanos* (*nos*) mRNA are localized to two opposing poles of the embryo to define the anteroposterior axis. For determination of the dorsoventral axis, a ventral signal is produced at the ventral perivitelline space to provide polarity for the axis. Establishment of the two body axes was once thought to be initiated independently (St. Johnston and Nüsslein-Volhard, 1992) but it is now considered to originate initially from a single signaling pathway. The signaling pathway depends on communications between both the germ cell and somatic cells of the egg chambers (reviewed by Anderson, 1995; Lehmann, 1995). During early oogenesis, an oocyte is produced by four synchronous cell divisions of a stem cell in the germarium with incomplete cytokinesis, in which actin-rich intercellular bridges, or ring canals, connect the posteriorly located oocyte and the remaining fifteen nurse cells (Figure 1.3). The ring canals allow transportation of the necessities for development from the nurse cells to the oocyte, whereas transcription is inactive. The oocyte-nurse cell cluster is covered by a layer of

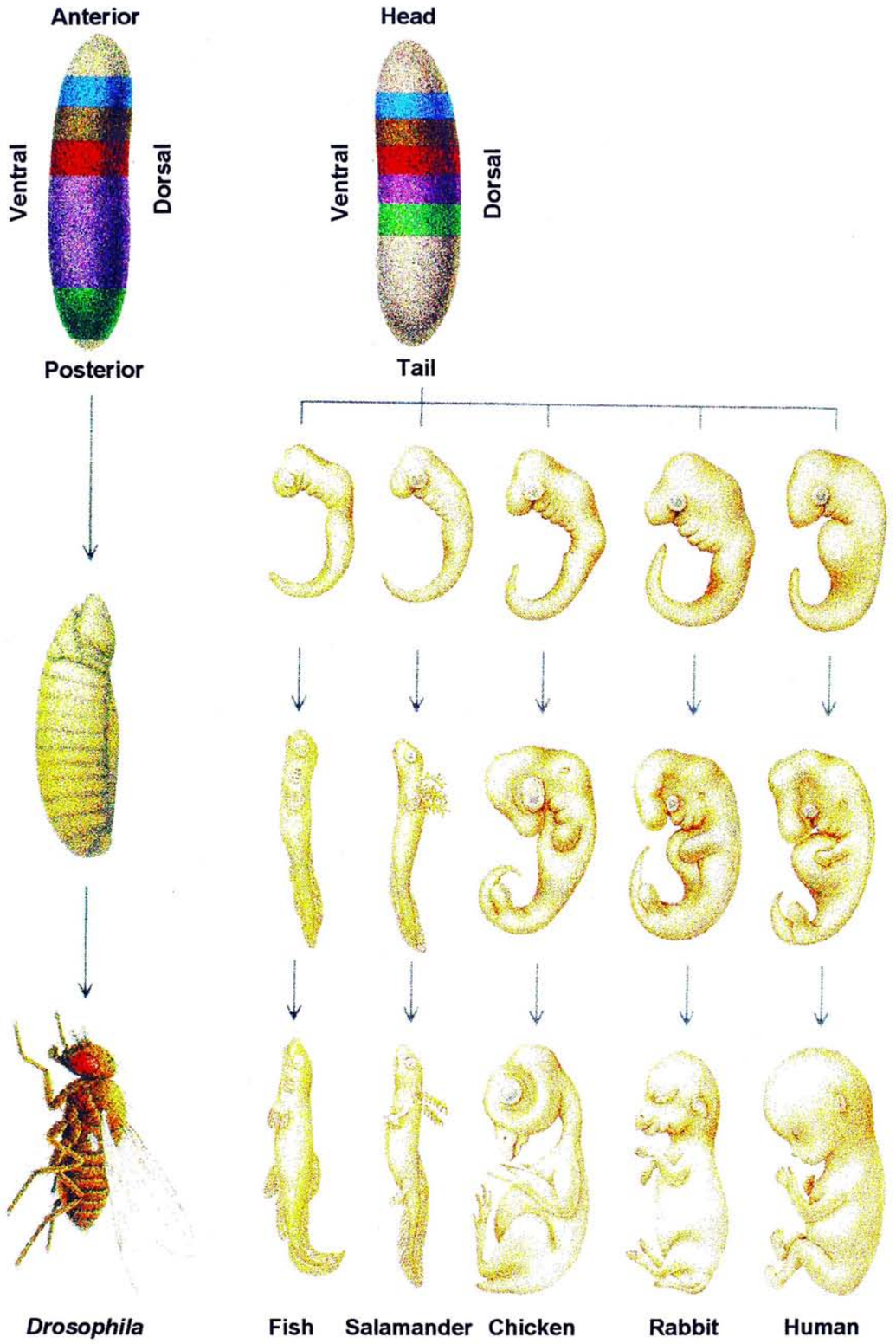


Figure 1.1 The basic body plans of animals.

Despite the differences in the developmental machinery and morphology among various classes of animals, they all share one common feature - they have two fundamental body axes, the anteroposterior axis and the dorsoventral axis. (Adapted from McGinnis and Kuziora, 1994)

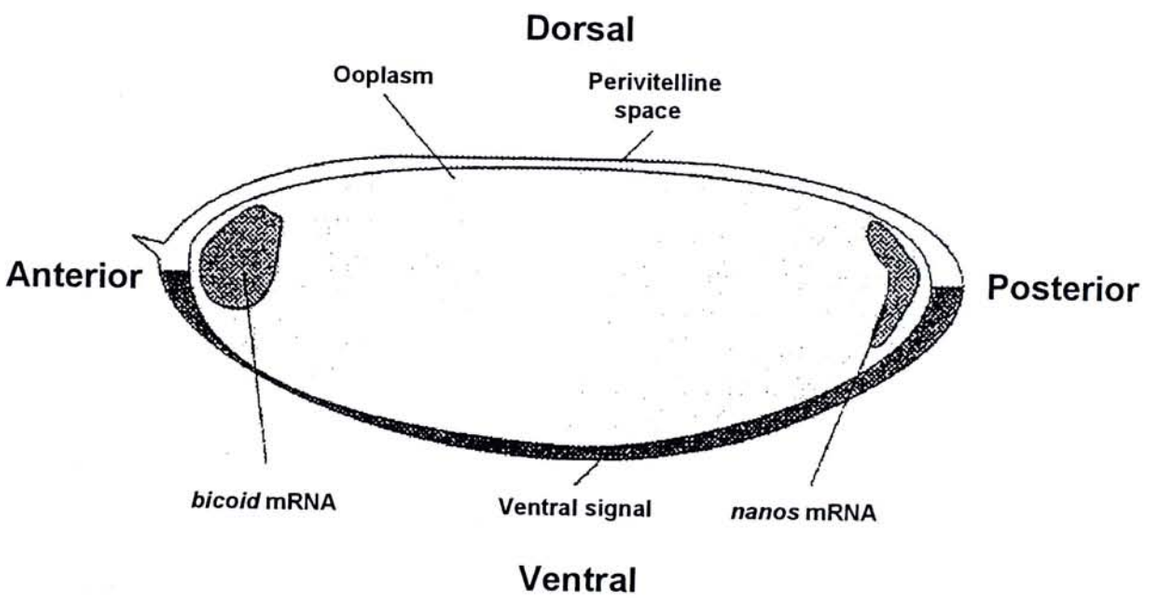


Figure 1.2 Body axis formation by asymmetrical localized signals in the *Drosophila* embryo.

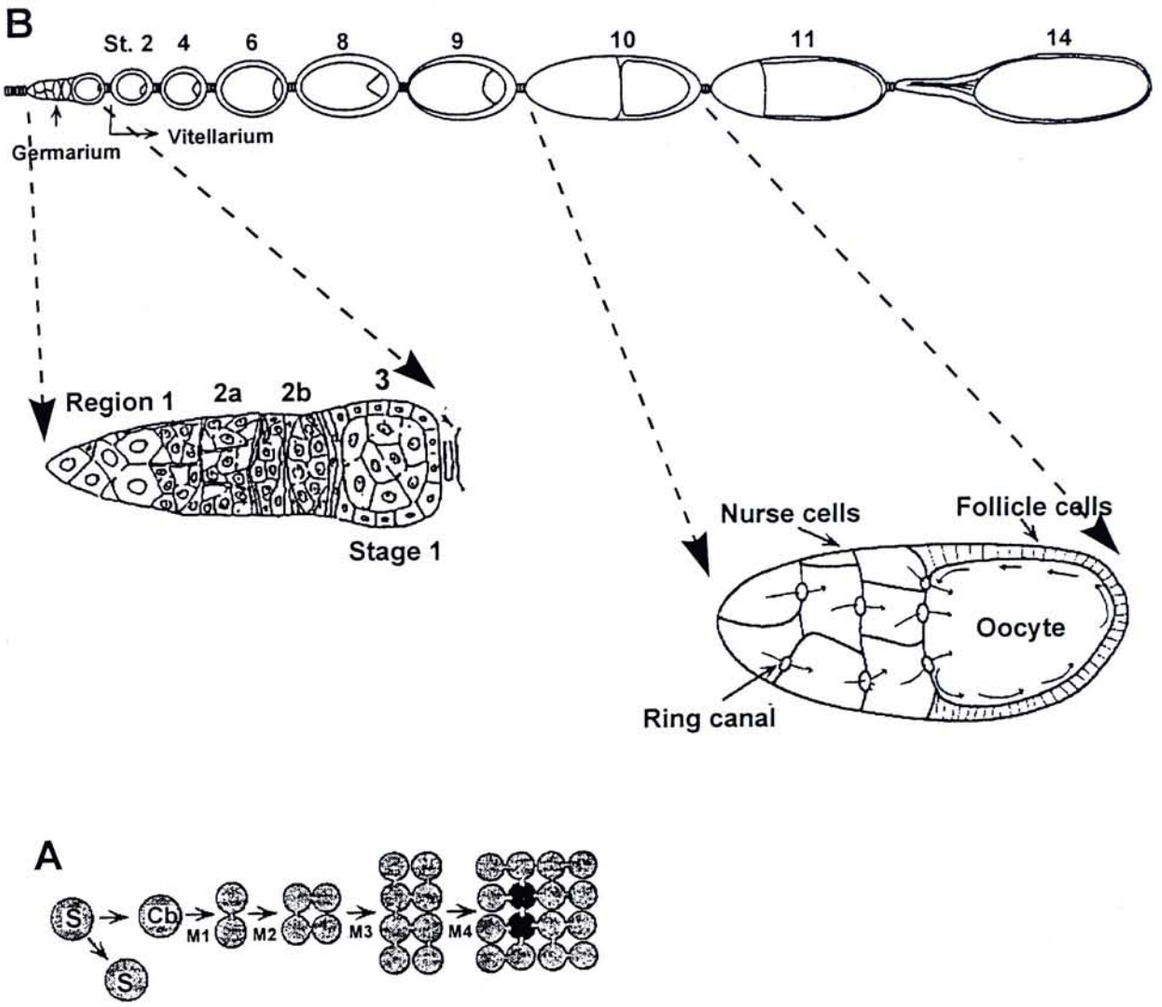


Figure 1.3 Formation of developing eggs from the germarium.

A. Oogenesis begins in region 1 **(B),** with the division of a germline stem cell (S) to produce a cystoblast (Cb) and regenerate a stem cell. The cystoblast then divides by four synchronous cell divisions (M1-M4) with incomplete cytokinesis into a 16-cell cyst in which cells are interconnected with actin-rich ring canals. One of the two cells (the black ones) with four ring canals adopts the cell fate of oocyte and takes a posterior position within the egg chamber. **B.** The oocyte is developed into a mature egg in a process called oogenesis which can be divided into different stages (King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993). Mature eggs are fertilized and hatched as embryos at the end of oogenesis. (Adapted from Cooley and Theurkarf, 1994)

somatic follicle cells which provide an asymmetry to the oocyte, where it is in contact with the nurse cells at the anterior on the one end and with the follicle cells at the posterior on the other end.

As the oocyte grows, a microtubule-organizing center (MTOC) located at the posterior pole of the oocyte extends microtubules into the nurse cells (Theurkauf et al., 1992, 1993; reviewed by Cooley and Theurkauf, 1994; St Johnston, 1995) (Figure 1.4). This microtubule network allows transportation of RNA and protein molecules, from the nurse cells to the oocyte, presumably via minus end-directed motors. At mid-oogenesis, there is a reorganization of polarity of microtubules in which the posterior MTOC degenerates and microtubules nucleates at the anterior pole of the oocyte. This reorganization, which is critical for the body axis specification, requires a signal from the posterior follicle cells. The transforming growth factor α (TGFα) homologue, *gurken*, and the epidermal growth factor receptor homologue, *DER/top*, are thought to provide the signal (González-Reyes et al., 1995; Roth et al., 1995; reviewed by Lehmann, 1995; Anderson, 1995). During early stages, the *gurken* RNA accumulates between the oocyte nucleus and the posterior pole, and the synthesis of gurken protein is thought to activate the DER/TOP receptor tyrosine kinase in the follicle cells which in turn leads to the expression of posterior polar follicle markers (reviewed by Schüpbach and Roth, 1994) (Figure 1.5). The two posterior polar follicle cells then signal back the oocyte to reorganize the polarity of microtubules. Protein kinase A (PKA) in the oocyte is a likely candidate to receive this signal from the follicle cells. The activation of PKA may function to destabilize the posterior MTOC and revert the polarity of microtubules. In the PKA mutants, failure in reorientation of the polarity of microtubules led to mislocalization of the maternal determinants *bicoid* and *oskar* RNAs (Lane and Kalderon, 1994; reviewed by St. Johnston, 1995; reviewed by Anderson, 1995), whose localization at the anterior pole and posterior pole, respectively, defines the anteroposterior axis (Frohnhöfer and Nüsslein-Volhard, 1986, 1987; Schüpbach and Wiechaus, 1986; Lehmann and Nüsslein-Volhard, 1986, 1987; Ephrussi et al., 1991; Kim-Ha et al., 1991). Therefore, the reorganization of microtubules is required for determination of the anteroposterior axis.

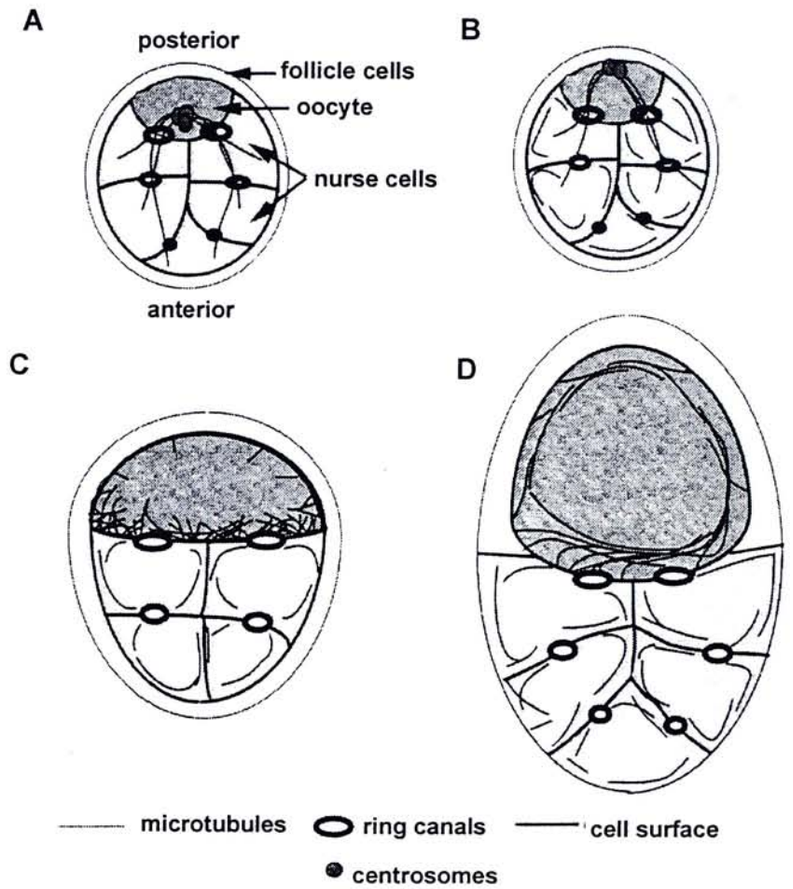


Figure 1.4 Microtubule reorganization during oogenesis.

A. Stage 1. An MTOC appears to localize at the anterior pole of stage 1 oocyte near the ring canals. **B.** Stages 2-6. The MTOC shifts to the posterior pole and extends microtubules into adjacent nurse cells. **C.** Stages 7-10A. The posterior MTOC is no longer detectable but high concentration of microtubules is detected at the anterior cortex of the oocyte. A gradient of microtubules is formed from the anterior end to the posterior end of the oocyte. **D.** Stage 10B to 12. Subcortical microtubule bundles assemble and ooplasmic streaming begins. The egg chambers are not drawn to scale. (Adapted from Theurkauf et al., 1992)

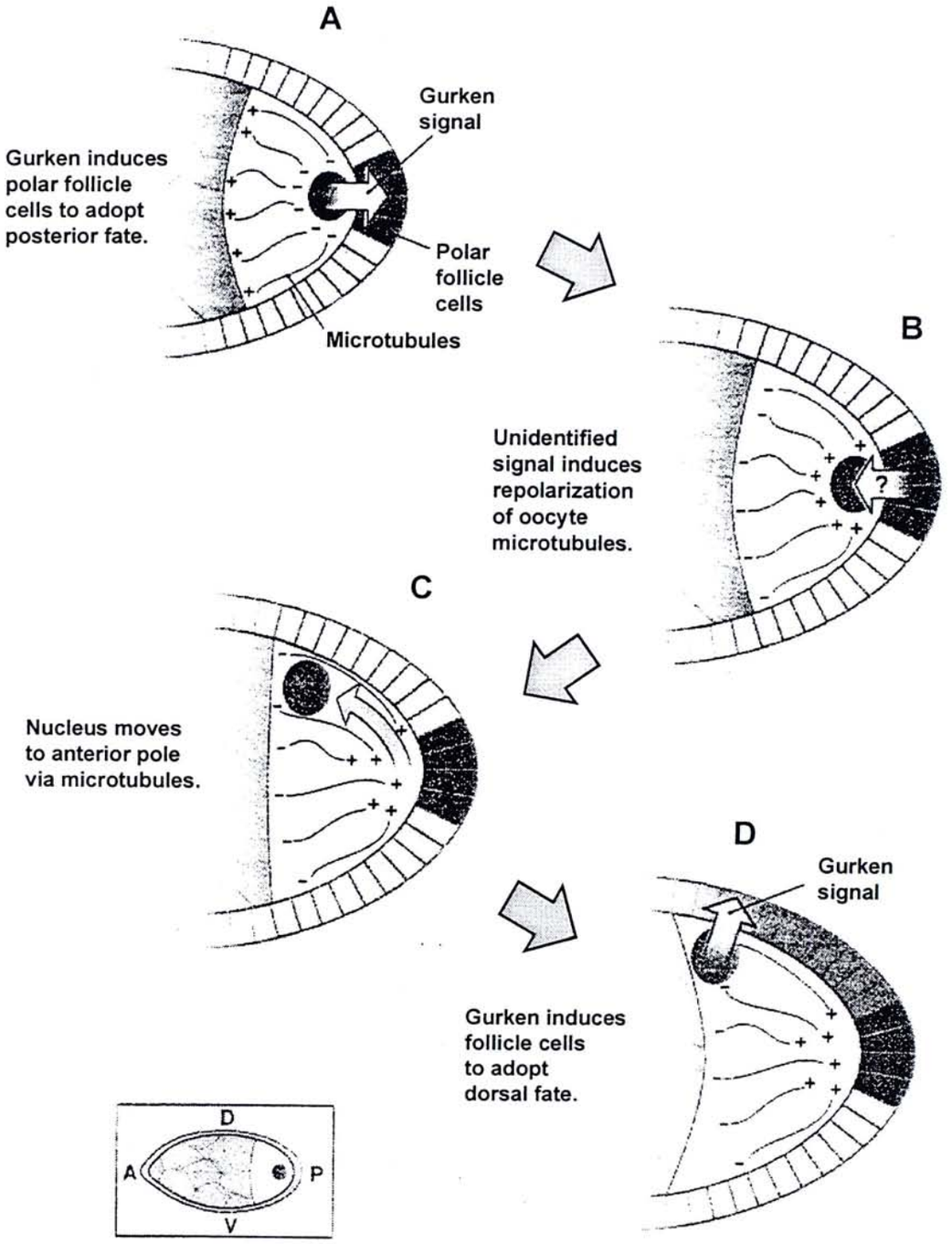


Figure 1.5 A model for the initiation of establishment of the two body axes by one single signal.

A. The *gurken* mRNA is associated with the nucleus which locates at the posterior pole of the oocyte at stages 3-6. Its expression induces the follicle cells to adopt posterior cell fates. **B.** The two posterior polar follicle cells then signal back to induce the reorganization of microtubules within the oocyte. **C.** The nucleus as well as the *gurken* mRNA move along the cortex toward the anterior in response to the repolarization of the microtubules. **D.** Once the nucleus reaches the anterior corner of the oocyte, *gurken* signals the overlying follicle cells to adopt dorsal cell fates. Thus the formation two body axes can be initiated by one single signal. (Adapted from Anderson, 1995)

The reorganization of microtubules is also essential for the establishment of the dorsal-ventral polarity. The oocyte nucleus seems to be always associated with the minus end of the microtubules (reviewed by Cooley and Theurkauf, 1994) and moves from the posterior to the anterior cortex of the oocyte during the reorientation of polarity of microtubules (Figure 1.5) (González-Reyes et al., 1995; Roth et al., 1995). The *gurken* mRNA, which remains associated with the oocyte nucleus, moves together with the oocyte nucleus to the future anterior-dorsal corner of the oocyte and thus defines the dorsal-ventral axis (reviewed by Schüpbach and Roth, 1994). The mechanism was thought to involve in the synthesis of gurken protein which activates the DER/TOP receptor in the overlying dorsal follicle cells. These dorsal follicle cells take a dorsal cell fates and this in turn defines the ventral follicle cell fates. The dorsal-ventral polarity is established by the activation of the TOLL receptor in the ventral oocyte membrane through the signal provided from the ventral follicle cells (reviewed by Morisato and Anderson, 1995).

1.2 Maternal genes are essential for development

The reorganization of microtubules allows subsequent localization of the maternal signals, *bcd* RNA and *oskar* RNA. These signals, which are not originated zygotically, are synthesized in somatic nurse cells and deposited to the developing egg during oogenesis. The basic organization and polarity of the two main body axes are defined and initiated by these signals. Only a small number of genes (about 30) have been identified to encode the maternal signals (Nüsslein-Volhard et al. 1987). These genes can be further divided into four groups on the basis of different parts of the body axes. One is the dorsoventral group genes which are responsible for specifying the dorsoventral axis of the embryo. The other three groups are the anterior, posterior and terminal genes which are necessary for defining the anteroposterior axis.

Most of our knowledge on these maternal genes come from the study of genetic screens designed to isolate maternal-effect mutations that affect the embryonic pattern (Gans et al., 1975; Schüpbach and Wieschaus, 1986; Nüsslein-Volhard et al., 1987). Although some of the eggs were normal in shape, the embryos produced by maternal-effect mutants showed defects with cuticular

pattern. Such defects were accounted as reduction or loss of some part of the body structure. Mutations in the anterior maternal genes resulted in the loss of anterior head and thoracic structures, while the posterior maternal mutations caused deletions of abdominal segments, and the terminal mutations produced defects in the unsegmented ends of the embryo, the acron and telson (Figure 1.6). For the anteroposterior axis, several mutations, *bicoid* (*bcd*), *exuperantia* (*exu*), *swallow* (*sww*), *staufen* (*stau*), *bicaudal* (*bic*), *Bicaudal-D* (*BicD*) and *Bicaudal-C* (*BicC*) (Frohnhöfer and Nüsslein-Volhard ,1986, 1987; Schüpbach and Weischaus, 1986; Gans et al., 1975; Zalokar et al., 1975; Mohler, 1977; Stephenson and Mahowald; Nüsslein-Volhard, 1977; Mohler and Wieschaus, 1985; reviewed by Nüsslein-Volhard et al., 1987) are responsible for the anterior defects, while *oskar* (*osk*), *vasa* (*vas*), *tudor* (*tud*), *staufen* (*stau*), *valois* (*val*), *nanos* (*nos*) and *pumilio* (*pum*) (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Weischaus, 1986; Boswell and Mahowald, 1985; reviewed by Nüsslein-Volhard et al., 1987) affect the posterior pattern, and *torso* (*tor*), *trunk* (*trk*), *torsolike* (*tsl*), *fs(1) polehole* [*fs(1) ph*] and *fs(1) Nasrat* [*fs(1) N*] (Schüpbach and Weischaus, 1986; Perrimon et al., 1986; Degelmann et al., 1986; reviewed by Nüsslein-Volhard et al., 1987) determines the terminal structures. The mutation *stau* produces defects in both anterior and posterior patterns and is classified into both groups.

1.3 Maternal gene *bicoid* is required for formation of anterior structures in the embryo

Mutations in one of the anterior maternal genes, *bicoid* (*bcd*), led to loss of head and thorax and duplication of telson at the anterior end of the embryos (Figure 1.7) (Frohnhöfer and Nüsslein-Volhard, 1986). Thus *bcd* is required for formation of the anterior pattern. Cytoplasmic transplantation experiments have revealed that the activity of *bcd* is localized at the anterior pole of the embryos (Frohnhöfer and Nüsslein-Volhard, 1986, 1987). This finding was also supported by other experimental evidence. For instance, transplantation of cytoplasm from the anterior pole of the wild type embryo to the same region of the *bcd* mutant embryo was shown to restore the normal phenotype, while the development of anterior structures were observed after the injection of the corresponding cytoplasmic

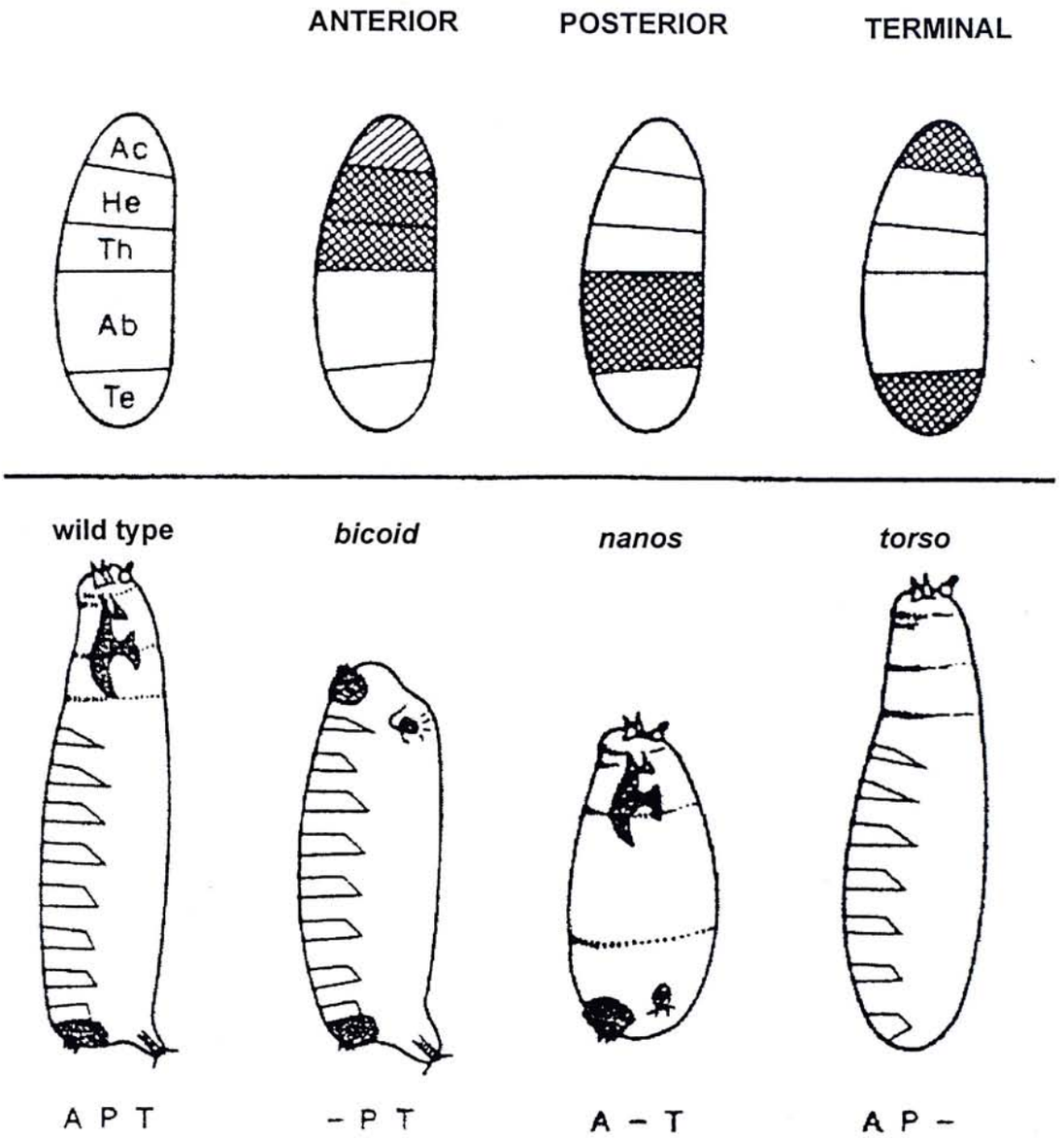


Figure 1.6 Embryonic pattern of maternal mutants.

Mutations that result in the loss of corresponding body structures. For the anterior maternal mutants, head and thoracic structures are lost, while abdominal segments are missing in the posterior maternal mutants, and unsegmented regions such as acron and telson are deleted in the terminal maternal mutants. (A, anterior structures, such as head and thoracic segments; P, posterior structures, abdominal segments; T, terminal structures, acron and telson; Ac, acron; He, head; Th, thorax; Ab, abdomen; Te, telson) (Adapted from St Johnston and Nüsslein-Volhard, 1992)

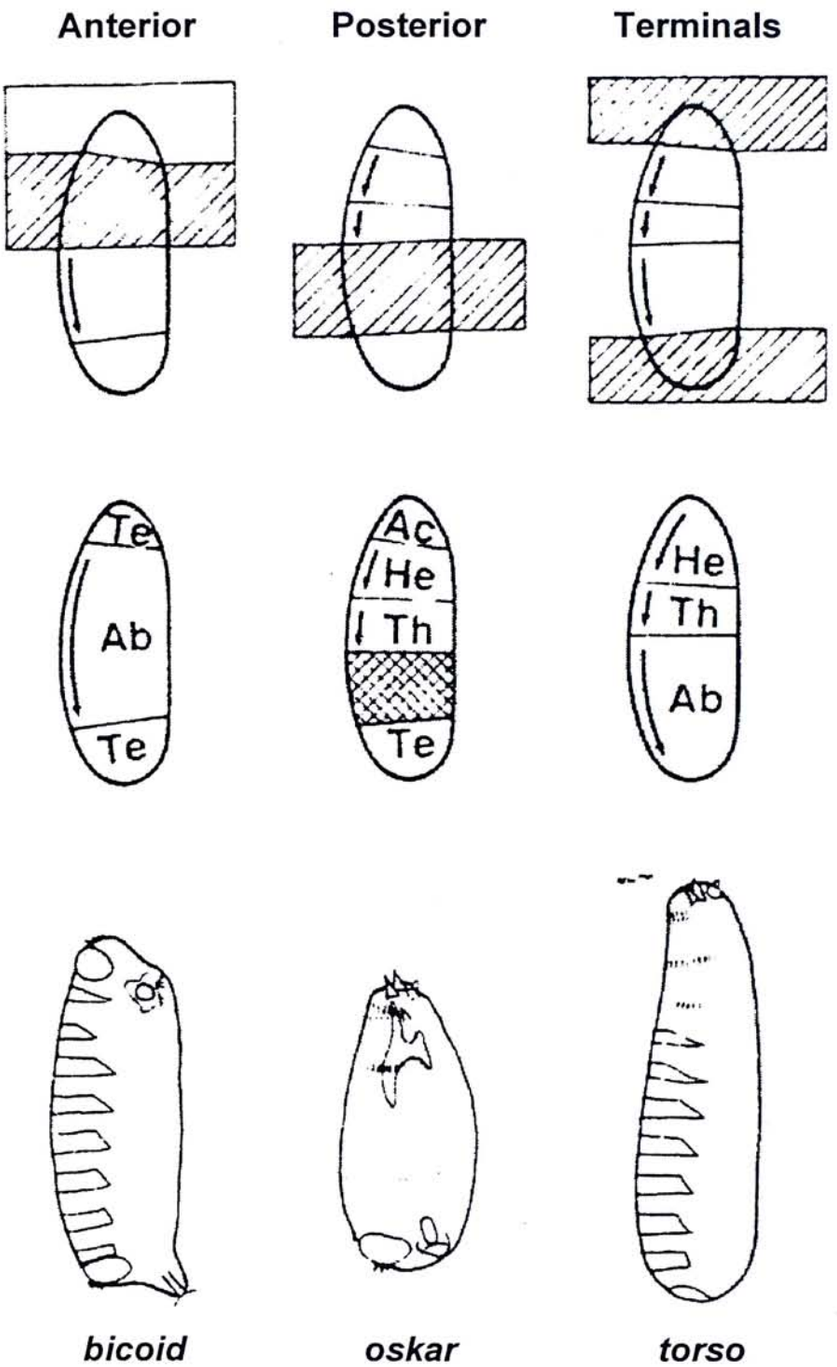


Figure 1.7 Defects in the maternal gene mutants.

In *bicoid* mutant, the head and thoracic structures are lost and the anterior acron is transformed into a telson. In *oskar* mutant, abdominal segments are lost while the acron and telson are missing in the *torso* mutants. The hatched rectangles in the upper row indicates that the normal origin of the regions are deleted. The second row shows the corresponding changes in the fate maps of the embryo while the lower row shows the cuticle defects of the larvae. Ac, acron; Th, thorax; He, head; Ab, abdomen; Te, telson. (Adapted from Nüsslein-Volhard et al., 1987)

homogenate at various positions along the anteroposterior axis of the embryo (Frohnhöfer and Nüsslein-Volhard, 1986, 1987).

1.4 Establishment of an anterior to posterior bcd protein gradient

The anterior structure inducing activity present in the cytoplasm of the anterior pole was in fact the gene products of *bcd*. In the embryo, *bcd* mRNA is localized in the cytoplasm at the anterior cortex, and is translated to produce an anterior to posterior concentration gradient of bicoid protein, which extends over the anterior two-thirds of the embryo (Figure 1.8) (Frigerio et al., 1986; Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988a; St. Johnston et al., 1989). This protein gradient determines the development of anterior structures in a concentration dependent manner in which changes in the *bcd* gene dosage led to complementary shifts in both the protein gradient and the anterior fate map (Driver and Nüsslein-Volhard, 1988b).

The bcd protein concentration gradient was produced by translation of and diffusion of bcd protein from the *bcd* mRNA source, and degradation of the bcd protein throughout the embryo. To produce a stable and nonlinear gradient of bcd protein, the rates of *bcd* mRNA translation, of diffusion and of dispersed proteolytic degradation should reach an appropriate balance (Driever and Nüsslein-Volhard, 1988a). For such a model, bcd protein should have a low stability. The presence of several PEST sequences (Driever and Nüsslein-Volhard, 1988a), which are present in some proteins of short half-life and are thought to be signals for degradation (Rogers *et al.*, 1986; Rechsteiner *et al.*, 1987), in bcd protein may reflect its low stability.

1.5 The bcd protein gradient regulates the downstream zygotic target genes in a concentration-dependent manner

Once the concentration gradient of bcd protein has been established in the embryo, bcd protein will act as transcriptional activators to regulate the genes downstream. In response to the bcd gradient as well as the gradient produced by the posterior determinants nanos, a group of zygotic target genes, which determine the

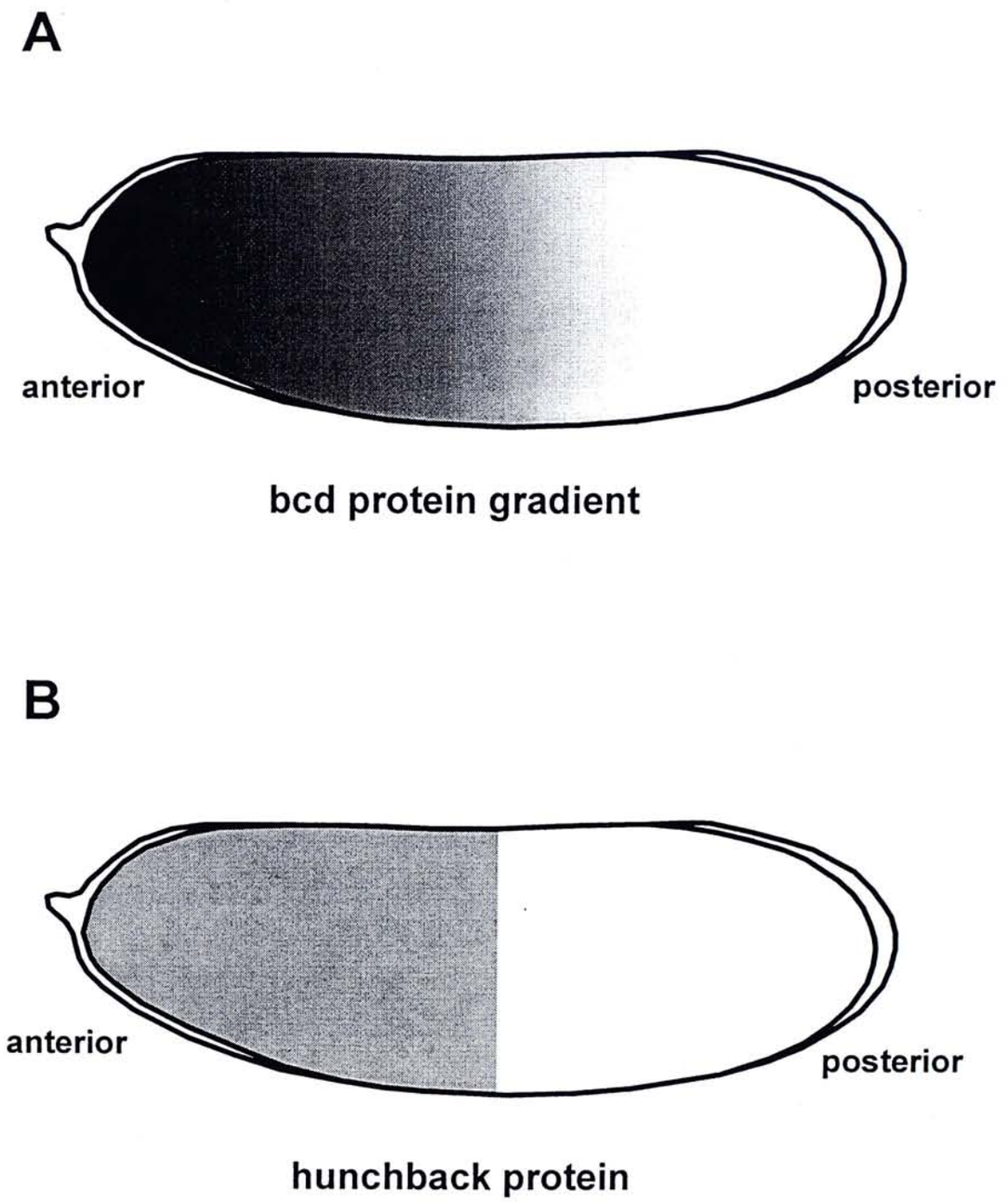


Figure 1.8 Expression of *bicoid* (*bcd*) and *hunchback* (*hb*).

A. bcd protein gradient is produced by translation of the anteriorly localized *bcd* mRNA and extends over the anterior two-thirds of the embryo. **B.** Translation of homogeneously distributed *hb* mRNA is activated by bcd protein at the anterior while it is inhibited by nanos at the posterior, producing uniform distribution of hb protein at the anterior half of the embryo.

pre-pattern of the embryo, are spatially expressed along the anteroposterior axis. This group of zygotic genes known as gap genes whose mutations result in elimination of particular regions of the embryo and create gap in the anterioposterior pattern (Ingham, 1988). Three principle members of gap genes, *hunchback*, *Krüppel*, and *knirps* (Nüsslein-Volhard and Wiechaus, 1980) are spatially expressed along the embryo from the anterior to posterior to provide the first subdivision of the embryo (Knipple et al., 1985; Tautz, 1987, 1988; Gaul et al., 1987; Pankratz et al., 1989). The gene products of the spatial gene expression contain DNA binding finger domains which act as transcriptional regulators. These transcriptional regulators further regulate the downstream segmentation genes to subdivide the body axis into different regions (Rosenberg et al., 1986; Tautz et al., 1987; reviewed by Struhl et al., 1989). The gap genes in turn regulate the spatial expression of pair-rule genes and segment polarity genes which subdivide the embryo into its segmental units which form the segmented pattern of the larvae (Figure 1.9). The homeotic genes further specify and direct the development of different body parts in segments (reviewed by Ingham, 1988) and thus the cell fates can be precisely defined along the anteroposterior axis of the embryo.

1.6 bcd protein acts as transcriptional regulators

The presence of a homeodomain within the bcd protein sequence suggests that it is a sequence-specific DNA binding protein and directly regulates zygotic target genes (Frigerio *et al.*, 1986; Berleth *et al.*, 1988). One of these target genes is the gap gene *hunchback* (*hb*; Tautz *et al.*, 1987) which is required for defining structures of thorax and part of the head (Nüsslein-Volhard and Wiechaus, 1980; Tautz *et al.*, 1987). In response to the bcd protein gradient, hb is expressed homogeneously in the anterior half of the embryo with a sharp posterior border (Figure 1.8) (Driever et al., 1989; Struhl et al., 1989). The zygotic expression of *hunchback* is directly regulated by the binding of bcd protein. There are five bcd protein binding sites within the 300 bp immediately 5' to the start site of *hunchback* and three of the sites in the promotor region are both required and sufficient for the activation of expression of *hunchback* (Driever and Nüsslein-Volhard, 1989a). This region can direct the bcd-dependent expression of a reporter gene in the anterior

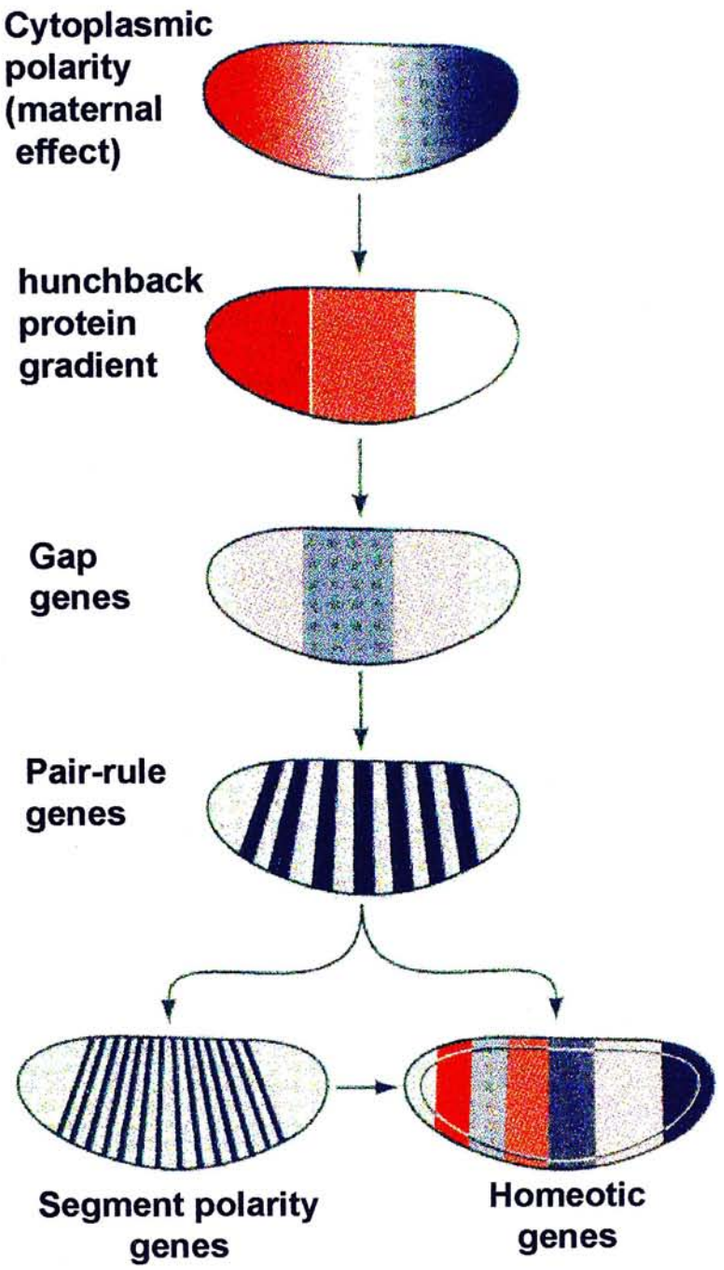


Figure 1.9 The hierarchy expression of different cascades of genes to refine the body pattern. (Adapted from Gilbert, 1994)

half of the embryo, suggesting that bicoid protein can bind directly to the region and activate the transcription of *hunchback* (Schröder *et al.*, 1988; Struhl *et al.*, 1989; Driever and Nüsslein-Volhard, 1989b).

The gene *hunchback* is one of the zygotic target genes which are regulated by bcd protein. The spatial expression of these genes is regulated by bcd protein in concentration-dependent manner. Changes in the maternal *bcd* gene dosage will produce complementary shift in both the bcd protein gradient and the spatial expression of zygotic target genes (Driever and Nüsslein-Volhard, 1989b, Ma et al., 1996). The bcd protein acts as a morphogen, with different threshold concentrations defining different anterior cell fates. It regulates the expression of the zygotic target genes by binding cooperatively to promoter regions (Struhl *et al.*, 1989; Driever and Nüsslein-Volhard, 1989b), similar to the binding of lambda repressor to its tripartite operator (Johnson et al., 1979; reviewed in Ptashne, 1986; Struhl et al., 1989). Bcd proteins with multiple DNA binding display positive cooperativity in the binding of target DNAs (Ma et al., 1996). In the cooperative binding process, a sigmodial binding curve was observed indicating that an on- and off- state of transcription is allowed within a narrow range of bcd protein concentrations (Driever and Nüsslein-Volhard, 1989a, 1989b). In such a model, bcd protein can bind multiple binding sites at concentration above certain threshold but cannot bind to any of the sites at a lower concentration at positions slightly more towards the posterior. Thus, genes, such as *hb*, with high affinity bcd binding sites will require low bcd concentrations to be activated and expressed in large anterior regions; while genes, such as *orthodenticle* (Finkelstein and Perrimon, 1990), *buttonhead* (Cohen and Jürgens, 1990; Wimmer et al., 1993), and *empty spiracles* (Dalton et al., 1989), with low affinity of the sites are activated only at higher concentrations and expressed in smaller anterior domains (reviewed by Ma et al., 1996). Therefore, a gradient of bcd protein is transformed into discrete domains of expression of zygotic target genes, leading to the formation of various distinctive regions of the embryo (Driever and Nüsslein-Volhard, 1989a, 1989b; Struhl et al., 1989).

Recent evidence has suggested that *hb* is not only responsible for defining the anterior structures but also plays the role as a morphogen for the determination of the polarity of the embryo. This *hunchback* gene is expressed both maternally

and zygotically. The proper spatial expression of the zygotic hb and all known bcd target genes requires synergistic activation by both maternal *hb* and *bcd* (Simpson-Brose et al., 1994). The expression of hb is necessary for specifying the correct long range polarity of the embryo. The maternal hb has a dose-dependent effect on the spatial expression of the gap genes *Krüppel*, *knirps*, and *giant* (Hülskamp et al., 1990; Struhl et al., 1992). Therefore, the anterior pattern of the embryo is not simply determined by a single morphogen gradient but rather a combination of multiple morphogens.

1.7 The anterior localization of *bcd* mRNA

The gradient of bcd protein concentration is transformed into spatial expression of downstream development genes along the anteroposterior axis. However, *bcd* mRNA must have been first localized to the anterior pole before this gradient is produced. Then how does this maternal mRNA get localized to its destination? The mechanism of this process has not been completely understood. The localization of *bcd* mRNA begins at oogenesis and can be distinguished into four phases according to St. Johnston et al, 1989 (Figure 1.10). The *bcd* mRNA begins to concentrate at the oocyte at stage 5 (stages according to King, 1970; Mahowald and Kambysellis, 1980; Spradling 1993). Through stage 6 to 9 of oogenesis (the first phase), *bcd* mRNA accumulates in a ring at the anterior end of the oocyte. In stages 9 to 10a follicles (phase 2), *bcd* mRNA localizes at both the anterior end of the oocyte and apical regions of the nurse cells. In stages 10b to 11 (the third phase), all *bcd* mRNA localizes to the anterior cortex while it localizes to a slightly dorsal spherical region at the anterior pole between stage 12 and egg deposition (phase 4).

1.8 Components required for the localization of *bcd* mRNA

1.8.1 *cis*-acting elements

This localization process of *bcd* mRNA was found to be dependent on several elements. One of the elements is a *cis*-acting sequence which is responsible for the localization. A discrete portion within the 3' untranslated region of the bcd mRNA is necessary for its anterior localization (Macdonald and Struhl 1988). This

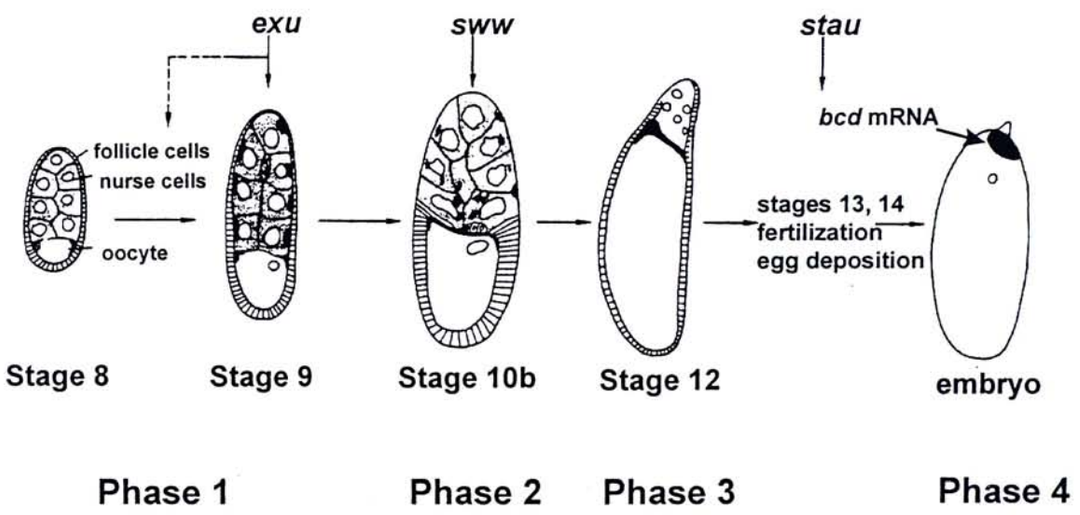


Figure 1.10 Four phases of localization of *bcd* mRNA.

The four phases of localization can be disrupted in several maternal mutants, *exu*, *sww*, and *stau*, and thus the three genes are involved in localization of *bcd* mRNA in a stepwise manner. (Adapted from St Johnston et al., 1989)

region is composed of 625 nucleotide at the 3' untranslated region (UTR) that is required for the localization and is sufficient to confer localization of a heterologous transcript to the anterior pole. It was also suggested that this sequence might form extended secondary structure which presented binding sites for its localization. Analysis of the 3' UTR of seven *Drosophila* species showed certain degree of homology (Macdonald, 1990). The primary sequences of the six species were up to 50 % divergent from the *D. melanogaster* gene, but they showed patchy homology throughout most of the region. Furthermore, all sequences of the seven species can potentially form a large stereotypic secondary structure (Figure 1.11), indicating that they were highly conserved in secondary structures despite divergence of the primary sequences. Besides, the 3' UTR showed functional conservation. The 3' UTR from three evolutionarily distinct species were tested interspecifically for *bcd* mRNA localization in *D. melanogaster* and each of them was found to be functionally interchangeable (Macdonald, 1990). Thus these 3' UTR are both structurally and functionally conserved and are essential for *bcd* mRNA localization.

1.8.1.1 BLE1 at 3' UTR directs localization of *bcd* mRNA

Further investigation on a series of *bcd* transgenes carrying small deletions in the 3' untranslated region had revealed that a *bcd* localization element 1 (BLE1) was responsible for early steps of localization (Macdonald et al., 1993). The BLE1 contained a sequence of 50 nucleotides and a transgenic mutant lacking this sequence had lost all the early localization. In addition, some other deletions within the 3' untranslated region were found to impair localization, suggesting that more than one localization elements would be present. Each of these elements may act on different steps of the localization process. One comparable example is the multiple localization elements in the 3' untranslated region of the *oskar* mRNA, with different elements mediating different steps in its localization to the posterior pole of the oocyte (Kim-Ha et al., 1993, reviewed by Macdonald et al., 1993).

Recent finding showed that another element, exl protein was involved in *bcd* mRNA localization process. It was demonstrated that there was a correlation between *in vitro* exl binding and one phase of *in vivo* localization directed by BLE1, implicating exl in that localization event (Macdonald et al., 1995).

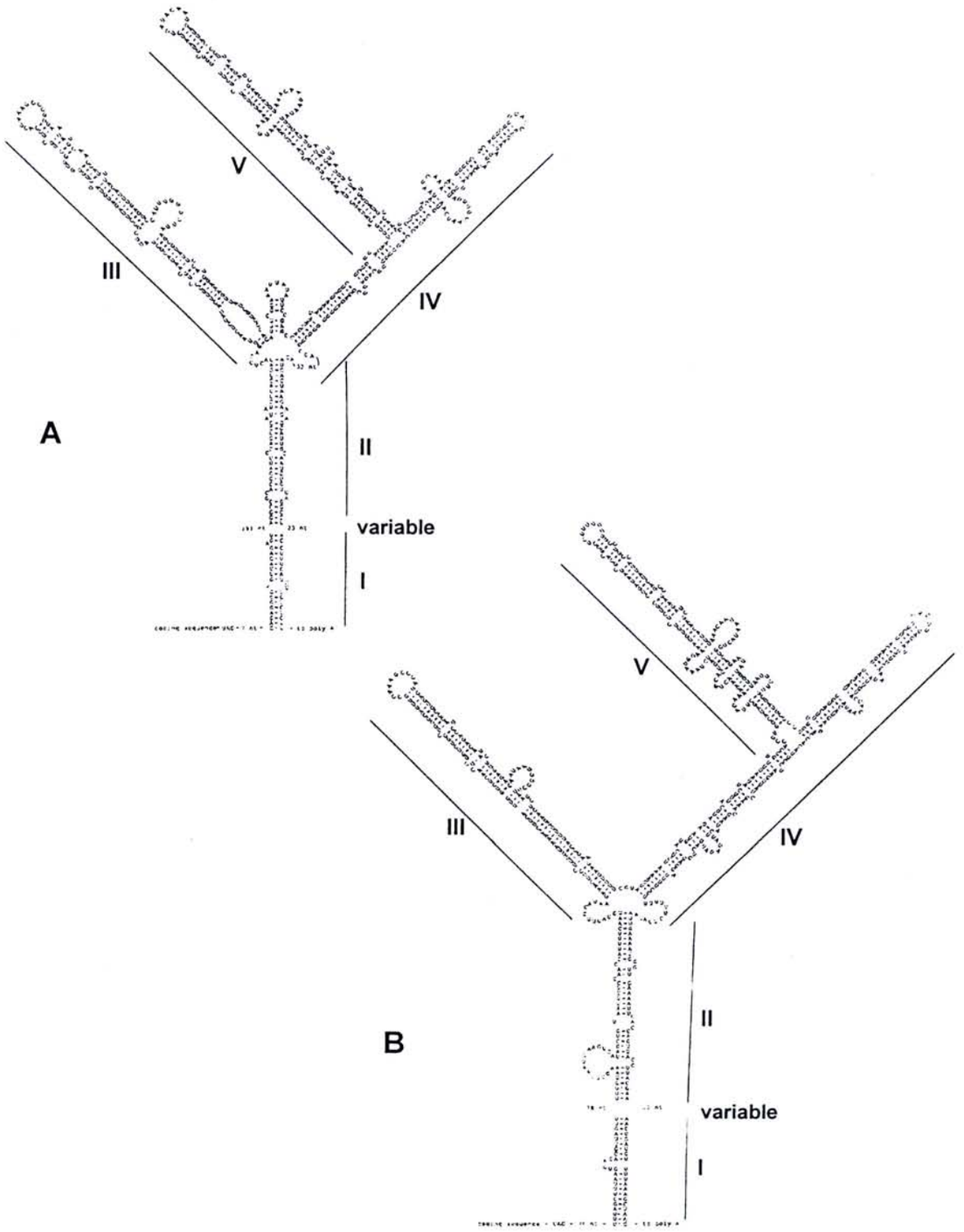


Figure 1.11 The conservation of the stereotypic secondary structure of the 3' untranslated regions (UTR) of bcd mRNA from two *Drosophila* species.

A. Predicted structure of *D. melanogaster*. **B.** Predicted structure of *D. virilis*. The 3' UTR of the two species are predicted to form conserved secondary structures with five major stems and a variable region. (Adapted from Macdonald, 1990)

A simple model of localization of *bcd* mRNA was proposed that once *bcd* mRNA is transcribed in the nurse cells, it is recognized and trapped after passing from the nurse cells to the anterior end of the developing oocyte through cytoplasmic bridges (Macdonald and Struhl 1988). In this model, certain receptors anchoring on structural components of the oocyte must be present to recognize and bind the *bcd* mRNA. These receptors do not need to be localized since the polarized entry of *bcd* mRNA already allows the anterior localization. Nevertheless, this model does not agree with other observation on relocalization of *bcd* mRNA after the treatment with microtubule destabilizing drugs (Pokrywka and Stephenson, 1991). Eggs treated with nocodazole, a microtubule destabilizing drug, had their *bcd* mRNA released from the anterior cortex and dispersed into the oocyte cytoplasm. After rinsing to remove the drug, most of *bcd* mRNA was relocalized at the anterior cortex. Although some *bcd* mRNA was mislocalized along the cortex at more posterior positions, higher concentrations of *bcd* mRNA were always present at the anterior tip. This observation indicated that the drug-released *bcd* mRNA could be relocalized to the anterior end after removal of the drug. Therefore, the localization of *bcd* mRNA is unlikely to involve simple transporting and trapping mechanisms. The localization of *bcd* mRNA must depend on other mechanisms and may require additional components.

1.8.2 *Trans*-acting elements

1.8.2.1 *exuperantia*, *swallow* and *staufen* are necessary for localization of *bcd* mRNA

There are several *trans*-acting elements responsible for *bcd* mRNA localization. Three anterior maternal genes, *exuperantia* (*exu*), *swallow* (*sww*) and *staufen* (*stau*) (Gans *et al.*, 1975; Schüpbach and Weischaus, 1986) are involved in the localization process (Frohnhöfer and Nüsslein-Volhard, 1987; Stephenson *et al.*, 1988; St. Johnston *et al.*, 1989). Mutations of these three genes resulted in the loss of anterior structures (Figure 1.12). Defects in localization of *bcd* mRNA were observed in all of these mutants (Figure 1.13). According to St. Johnston *et al.* (1989), *exu* mutants display the earliest defects in the localization among the three mutations. The initial localization of *bcd* mRNA during stages 5 to 7 is normal relative to the wild type. From stages 8 to 9, *bcd* mRNA is localized at the anterior

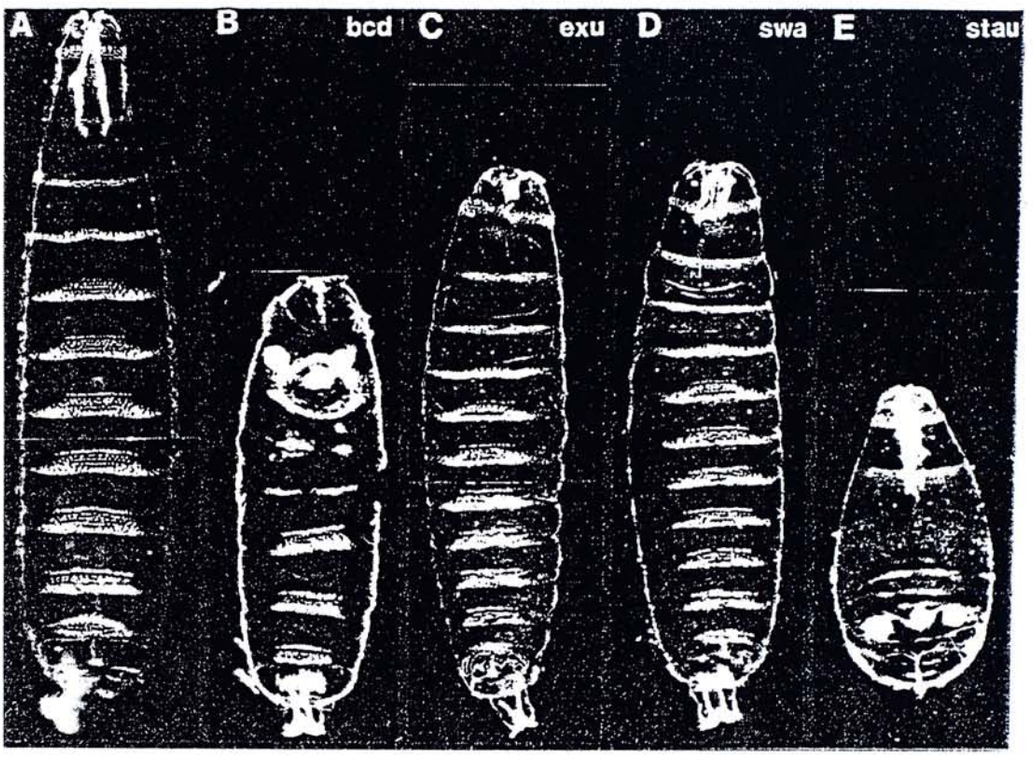


Figure 1.12 Cuticle patterns of maternal gene mutants showing loss of the anterior pattern in larvae.

A. wild type. **B.** bcd^{E1}, a strong *bcd* allele. Head and thoracic structures are replaced by a duplicated telson and the anterior abdomen is defective. **C.** *exu* mutant, and **D.** *sww* mutant shows loss of head structures. **E.** *stau* mutant displays anterior as well as abdominal defects. (Adapted from Driever and Nüsslein-Volhard, 1988b)

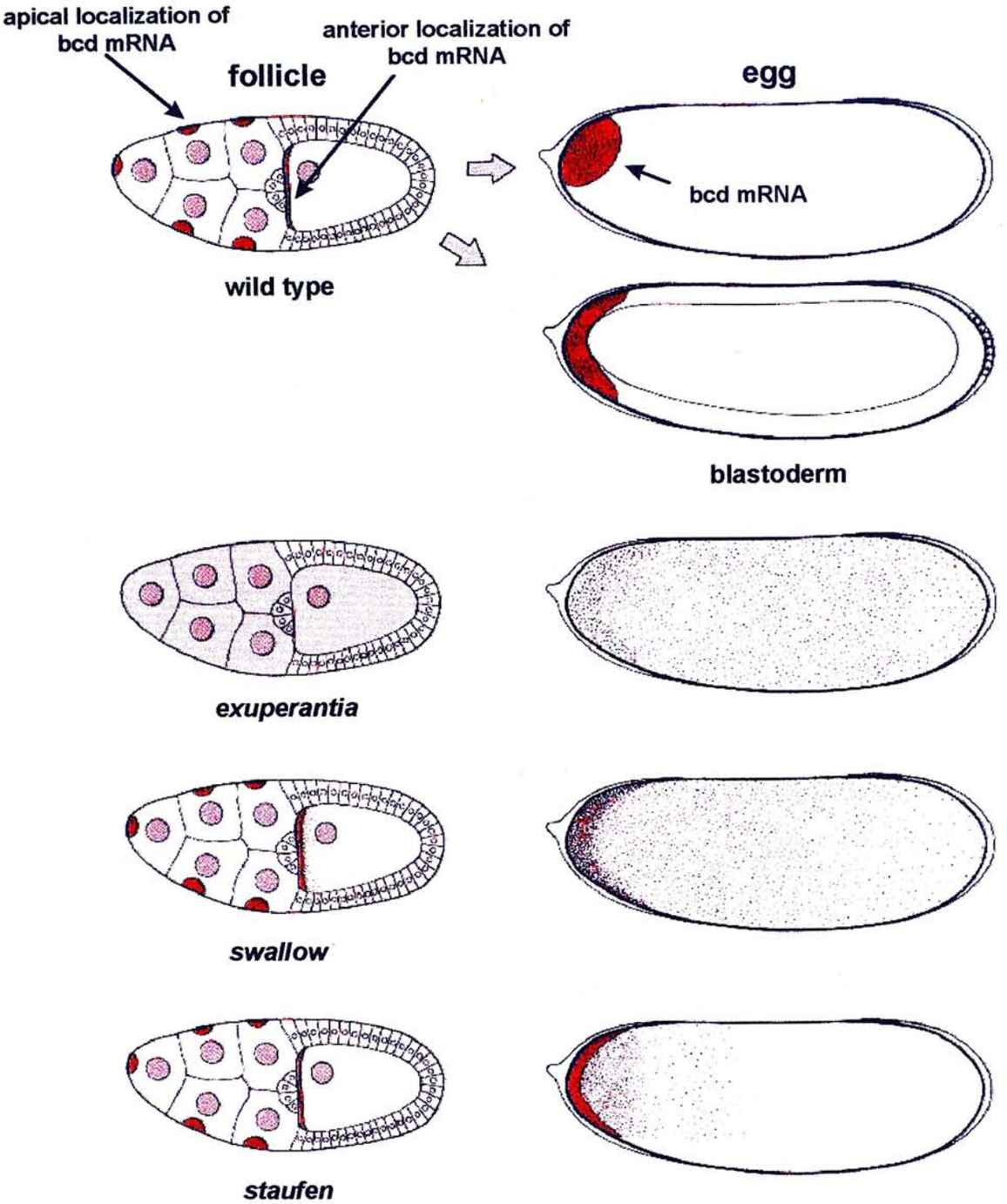


Figure 1.13 Defects in localization of *bcd* mRNA of the maternal gene mutations.

In the three mutants, *exu*, *sww* and *stau*, *bcd* mRNA is not tightly localized to the anterior pole of the oocyte. The *exu* mutants display the earliest defects in the localization and result in loss of the anterior localization in the oocyte and the apical localization in the nurse cells. (Adapted from Lawrence, 1992)

cortex of the oocyte but the mRNAs are more diffused as opposed to the tight localization as seen in the wild type. Furthermore, *bcd* mRNA is localized neither at the anterior pole of the stage 10 oocyte nor to the apical regions of the nurse cells in the mutants. Instead, it shows a uniform distribution within the oocyte and nurse cells.

In *sww* ovaries, the localization of *bcd* mRNA is entirely normal up to stage 10a. During stages 10b and 11, the anterior localization of *bcd* RNA appears to slip posteriorly and become more diffused. By stage 12, all *bcd* mRNA is released from the cortex and form a shallow anterior to posterior gradient. In *stau* mutants, *bcd* mRNA seems to have normal localization up until stage 12 and it is released from the anterior pole after this stage. The localization of *bcd* mRNA forms a gradient in the early embryos. All the mutations cause a loss or shallower gradient of bcd protein and account for the loss of the anterior body structures in the embryos.

1.8.2.2 exu protein is an absolute requirement for the localization

The three maternal genes are required in the localization of *bcd* mRNA in a stepwise manner (Figure 1.10) (St Johnston et al., 1989). As *exu* mutations produce an early defect in the localization, it seems to play an important role for localization of *bcd* mRNA during oogenesis. The *exu* gene seemed to be not necessary for the maintenance of the localization during late stages of oogenesis and early embryogenesis but rather for the establishment of the localization in the developing oocyte (Macdonald et al., 1991; Marcey et al., 1991). Other than in oogenesis, *exu* was found to also function in spermatogenesis (Hazelrigg et al., 1990) and several *exu* alleles were analyzed to be male sterile. The *exu* gene encodes overlapping sex-specific transcripts during oogenesis and spermatogenesis. Three kinds of mRNA are transcribed from *exu* gene. One is a major female germline-specific 2.1 kb transcript and the second one is a male germline-specific 2.9 kb transcript which overlaps with the female one, while the third one is rare 2.5 kb transcript which is expressed in somatic cells (Marcey et al., 1991). Three exons in *exu* mRNA comprise an open reading frame (ORF) of 1596 nucleotides. Translation of the *exu* ORF yields a protein of 532 amino acids with a predicted molecular weight of 57981 Daltons. The exu protein has a predicted pI of 10.12 and contains several basic, arginine-rich stretches (Marcey et al., 1991).

1.8.2.3 Potential functions of exu based on the coding sequence

Database search showed that exu protein has no significant homology to any known proteins (Macdonald et al., 1991; Marcey et al., 1991). However, by searching for common functional motifs, a PEST domain, which is thought to confer susceptibility to rapid proteolysis, was located at residues 435-450 (Marcey et al., 1991). Besides, the analysis of a mutant exu^{PJ42} revealed that a basic residue, $arginine^{339}$ of exu protein is important for *exu* function in females. Furthermore, there are no previously defined RNA recognition motifs (Bandziulis *et al.*, 1989; Query *et al.*, 1989) found in the sequence of exu protein but the presence of certain ribonucleoprotein (RNP)-like motifs (Marcey et al., 1991) may reflect potential interactions of exu protein to nucleic acids. In fact, colocalization of *bcd* mRNA and exu protein was observed at some stages during oogenesis (Macdonald et al., 1991). As the colocalization occurred only at early stages (stages 5-10b) but not in late stages (after stage 10b and embryo), the interaction between *bcd* mRNA and exu protein must be transient and indirect. No exu protein is detectable in the embryo, where *bcd* mRNA is tightly localized at the anterior cortex, indicating that exu protein acts in the initial steps of localization but does not play for a persistent role (Macdonald et al., 1991). If exu protein does bind *bcd* mRNA in its role in *bcd* mRNA localization, exu should have certain recognition motifs for its binding. However, the recognition may be very specific for *bcd* mRNA binding and so it is possible that exu contains a previously unknown sequence for RNA binding. Alternatively, exu may not directly interact with *bcd* mRNA but may link to *bcd* mRNA by intermediate components.

The absence of exu protein in late oocytes (stages 10 onwards) and the embryo indicates that *exu* must function in the early oogenesis (Marcey *et al.*, 1991). As the localization of *bcd* mRNA persists through the stages where no exu protein presents, exu protein is unlikely to serve as a molecular bridge between *bcd* mRNA and the structural components of the oocyte. Instead, exu protein may either activate a component of the oocyte to bind *bcd* mRNA, or may act as a transient docking protein which connects *bcd* mRNA to its binding targets or may modify *bcd* mRNA to allow subsequent binding (Marcey *et al*, 1991).

1.8.2.4 Microtubules dependence of the localization

Another trans-acting element necessary for *bcd* mRNA localization is a cytoskeletal component, microtubules. Several biological processes involving cytoskeletal elements in other systems have been already investigated. In *Caenorhabditis elegans*, P-granules are segregated into the posterior cell at the first cleavage by a mechanism that requires microfilament function (Strome and Wood, 1983; Hill and Strome, 1988), while in Xenopus oocytes, both microtubules and microfilaments are required for the localization of *Vg1* RNA to the vegetal cortex (Yisraeli *et al.*, 1990).

Cytoskeletal components have long been known to play a role in localization of developmental determinants. It has been shown that microtubules are absolutely required for localization of *bcd* mRNA. Drugs that depolymerize microtubules perturbed all aspects of *bcd* mRNA localization, suggesting that the cortical cytoskeleton is an important element for the mechanism of localization (Pokrywka and Stephenson, 1991). The study using destabilizing drugs, nocodazole, colchicine and tubulozole-C revealed that all stages of anterior localization of *bcd* mRNA in the oocytes (Pokrywka and Stephenson, 1991; Kwan and Luk, unpublished; also see section 3.2) as well as the characteristic apical localization at nurse cells were lost. The observation suggested that microtubules are necessary for both the initial localization of *bcd* mRNA and the maintenance of its localization. Further study showed that the egg chambers recovered from drug treatment had their *bcd* mRNA, at least mostly, relocalized to the anterior margin (Pokrywka and Stephenson, 1991), suggesting that *bcd* mRNA is localized only in the presence of microtubules. However, excessive microtubules polymerization also altered *bcd* mRNA localization. Treatment with taxol resulted in ectopic localization in which *bcd* mRNA was dispersed within the oocyte and concentrated in aggregates at the lateral cortex as well as around the oocyte nucleus (Pokrywka and Stephenson, 1991). Therefore, microtubules are involved either directly in localizing *bcd* mRNA or indirectly in maintaining the integrity of other cytoskeletal functions required for the localization.

Pokrywka and Stephenson (1994) provided further evidence that both *bcd* mRNA and exu protein were associated with microtubules. By cellular fractionation, both *bcd* mRNA and exu protein were found to be associated with a

detergent-insoluble fraction and their associations could be released by microtubule-destabilizing drugs. This revealed that both *bcd* mRNA and exu protein were associated with components, presumably microtubules in the detergent-insoluble fraction. However, evidence which showed a direct association of exu protein and microtubules is lacking.

1.8.2.5 Microtubule polarity directs localization of *bcd* mRNA

Cell biological study provided further insight into the mechanism of localization of RNA of maternal genes by microtubules organization. The distribution of microtubules during oogenesis provided a simplified model on how microtubules direct RNA localization. As discussed in section 1.1, the reorganization of microtubule distribution is essential for localization of *bcd* and *osk* mRNAs. In the PKA mutants, the microtubule distribution is as normal as the wild type in the early egg chambers (stages 1-6), but the MTOC persists at the posterior end, in addition to the microtubule nucleating region at the anterior end of the oocyte in stages 7 and 8 egg chambers (Figure 1.14). At the same stages, ectopic localization of RNAs, in which a transient accumulation of *bcd* mRNA at the posterior in addition to the normal anterior localization, and the concentration of *osk* mRNA in the middle instead of localiztion to the posterior of the oocyte, was observed (Figure 1.14). The polarity of the microtubules and the localization of the RNAs suggest that the localization of *bcd* mRNA is associated with minus-end-directed motors, while the transportation of *osk* mRNA is associated with plus-end-directed motors. Thus a uniform polarized framework of microtubules is necessary for directing the asymmetrical distribution of the RNAs.

1.9 Functions of exu in localization of *bcd* mRNA

Although many components have been identified, the mechanism on how they work together to allow localization of *bcd* mRNA is still unknown. At least two components, exu protein and microtubules, are known to play an important role in the localization process. Nonetheless, the study of the functional domains of exu protein as well as its interaction with components such as microtubules will provide further insight into the localization mechanism of *bcd* mRNA, which

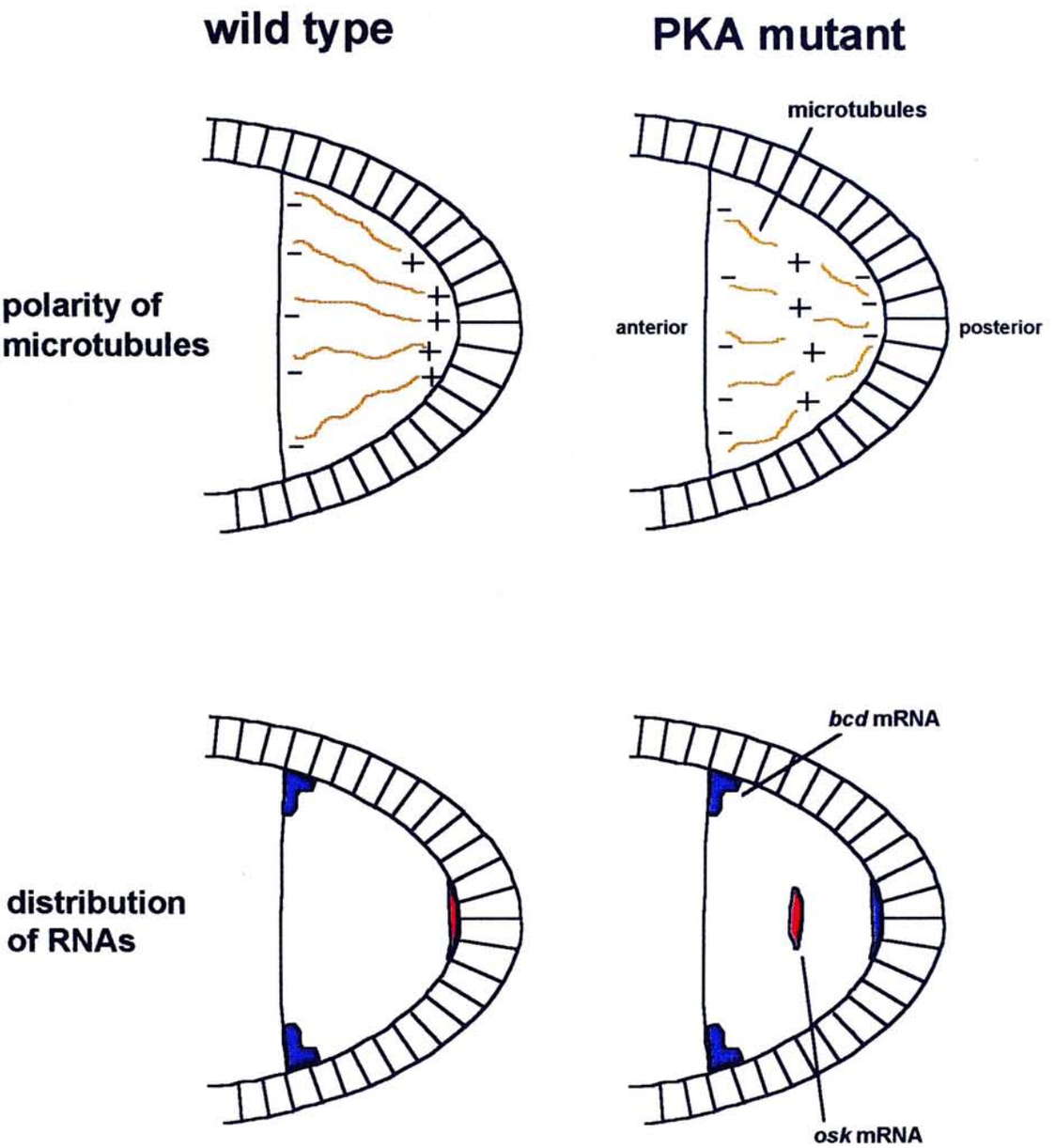


Figure 1.14 Polarity of microtubules and distribution of mRNAs in PKA mutants.

In PKA mutants, microtubule nucleating regions, which correspond to the minor ends of microtubules, were found at both anterior and posterior poles, creating an abnormal polarity of microtubules in the stage 7-8 oocyte. The *bcd* mRNA was localized at both poles of the oocyte, suggesting its transportation by minus-end directed motors, while *osk* mRNA was concentrated in middle of the oocyte, suggesting its association with plus-end directed motors.

represents one of the fundamental strategies in the determination of body axes in animals.

CHAPTER 2

Characterization of deletion mutants of *exu*

2.1 Introduction

The *exu* gene is absolutely required for the correct localization of *bcd* mRNA (Frohnhöfer and Nüsslein-Volhard, 1987; Stephenson et al., 1988; St Johnston et al. 1989). The exu protein colocalizes with *bcd* mRNA at mid-oogenesis (Macdonald et al., 1991) and it is likely to have domains that interact, either directly or indirectly, with *bcd* mRNA. Exploration and characterization of the functional domains will provide invaluable information on how a protein directs the localization of a polynucleotide which in turn determines the polarity of a developing egg.

Although the *exu* gene has been already cloned, the predicted protein sequence has neither homology to any characterized gene nor known common functional motifs (Marcey et al., 1991; Macdonald et al. 1991). Thus the protein does not provide any clue for its function. To locate the unknown functional domains of such a protein, the entire protein sequence would need to be examined and potentially important regions can be identified by molecular genetic analysis. By deletion analysis, potential functional domains of many different kinds of proteins, such as human interleukin-6 (IL-6) (Brakenhnoff et al., 1989), rat beta 1 thyroid hormone receptor (O'Donnell and Koenig, 1990), and bovine ras p21 GTPase activating protein (GAP) (Marshall et al., 1989) have been located and analyzed. In the case of *exu*, a series of deletion constructs which spanned almost the entire coding sequence were introduced into the germline by P-element transformation. Failure of these deletion mutants to complement the exu null allele would reflect the loss of structural/ functional regions and would facilitate the identification of potentially functional domains. Subsequent analysis of the functional domains would help us understand the function of *exu* and ultimately the localization of the anterior morphogen bicoid.

2.2 Construction of deletion mutants of exu

2.2.1 Materials and Methods

A molecular genetic approach was adopted to locate and determine which portion(s) of the exu protein is critical for its function. By taking advantage of the available restriction sites within the coding sequence, deletions were introduced into *exu* gene and resulted in eight different mutant constructs (Figure 2.1) (Kwan and Luk, manuscript in preparation). The constructs with deletions at the C-terminus (mutants Δ426-531 and Δ390-531) were tailed with a polylinker that contained termination codons in all three reading frames that allowed premature termination of translation. The exu proteins encoded by these constructs were thus truncated at the C-terminus. Other constructs were produced by restriction enzyme digestion and re-ligation either with (mutants Δ 54-193, Δ 194-375 and Δ 390-440) or without (mutants Δ 302-375, Δ 374-388 and Δ 442-503) linkers to restore the reading frame. All constructs were sequence-confirmed by dideoxy DNA sequencing.

The mutant constructs were introduced into the germline of w^{1118} flies by P-element mediated transformation (Rubin and Spradling, 1982). Transformants were identified by eye color, and balanced stocks were established for each independent lines of each mutation construct (Kwan and Luk, unpublished).

The transgenic constructs were tested for their ability to rescue the exu^{sc} maternal-effect lethality according to the method described by Macdonald et al. (1991). The exu^{sc} allele has a nonsense mutation at amino residue 53 of the predicted protein sequence (Macdonald et al., 1991). The mutation causes the premature termination of translation and is thus an exu protein null mutation. Eggs laid by females flies in homozygous exu^{sc} background are nonviable and cannot emerge as viable adults. The transgenic constructs were placed in the exu^{sc} background by appropriate crosses. Virgin females of the genotype exu^{sc} / exu^{sc} ; transgene construct / TM2 were crossed to wild type males to test for rescue of maternal-effect lethality. If adult progeny was emerged, the rescue test would be scored as positive; otherwise it would be scored as negative.

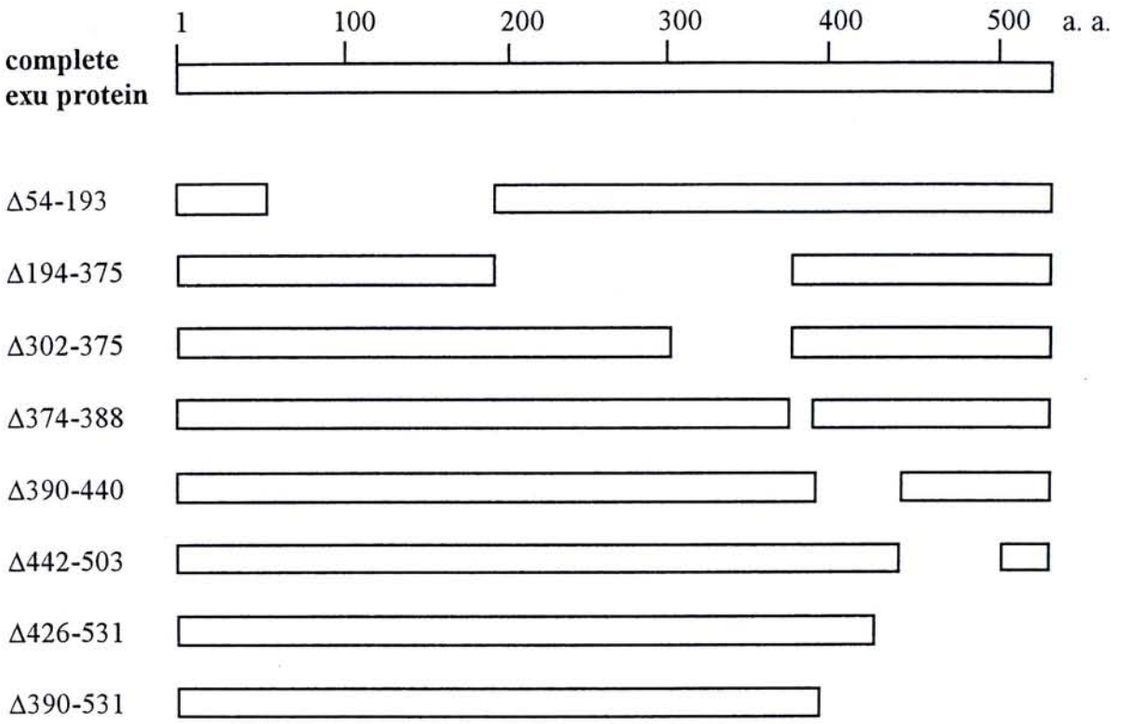


Fig. 2.1 Deletion mutants of *exu*. Restriction sites present in the exu sequence were used to remove unwanted fragments and the remaining fragments were re-ligated. Linkers were added before ligation to restore the reading frame in some deletion mutants and translation termination codons were introduced in those mutants with C-terminus being deleted. All the deletion mutants were sequence-confirmed the in-frame mutations. Open bars represent the exu protein sequence. Empty spaces between bars represent the various deletions. The two C-terminal deletion mutants (Δ 426-531, Δ 390-531) are represented by two truncated bars.

2.2.2 Results

At least two independent transgenic lines from each construct were tested for rescue. Of the eight transgenic constructs, one was found to be able to rescue the maternal-effect lethality, indicating that the mutant transgene, mutant Δ442-503 retained the full activity of the wild type *exu* gene. This deletion mutant, although having 62 internal amino acid residues deleted at the C-terminus, can replace the wild type *exu* gene to allow viable eggs to be produced. Thus this mutant was functionally indistinguishable from the wild type. None of the other seven transgenic constructs produced viable larvae indicating that they cannot replace the wild type *exu* gene (Table 2.1). However, mutant Δ 426-531 always produced one or two adult flies from a large number of eggs laid. Although viable progeny was observed, this line was considered as non-rescue as most of the eggs were not viable. By comparison of sequences between the rescue mutant Δ442-503 and the non-rescue mutant Δ426-531, the only difference was that the latter mutant had additional 16 amino acid residues deleted. These residues may play an important role in *exu* functions.

As mutant Δ442-503 still retained the full activity of the wild type *exu* gene, the deleted region within this mutant was dispensable for function. This region comprises 11.6 % of the whole exu protein and overlaps with part of the PEST domain located from residues 435 to 450. The PEST domain has been found in certain short-lived proteins and has been postulated to confer rapid proteolysis (Rogers *et al.*, 1986; Rechsteiner *et al.*, 1987). In mutant Δ442-503, only part of the PEST domain was deleted but the whole domain was removed in the non-rescue mutant Δ426-531. Therefore, it is possible that the removal of the whole PEST domain would account for the failure of rescue in mutant Δ426-531. Nonetheless, relationship between the presence of the PEST domain and the function of exu protein is at present unclear.

Table 2.1 Test for rescue of the maternal-effect lethality of the deletion mutants

Deletion mutants	Rescue
Δ54-193	-
Δ194-375	-
Δ302-375	-
Δ374-388	-
Δ390-440	-
Δ442-503	+
Δ426-531	-
Δ390-531	-

2.3 Analysis of exu protein in deletion mutants

The mutant constructs were tested if they expressed exu proteins *in vivo* during oogenesis. Total proteins from ovaries isolated from female flies homozygous for *exu* were analyzed by immunoblot.

2.3.1 Materials and Methods

2.3.1.1 Preparation of total ovary protein from the transgenic flies

Ovaries isolated from two-day-old female flies homozygous for exu^{sc} and contained at least one copy of the various transgenes were homogenized in 2X SDS sample buffer (SB) (2X: 62.5 mM Tris-HCl, pH 6.8, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, 0.005 % bromophenol blue) in a ratio of one pair of ovary to 10 µl of SB. Content of the ovary extracts was separated by SDS-PAGE and the presence of exu protein was detected by western blot analysis using an anti-exu antibody. The anti-exu antibody was a polyclonal antibody that allowed different epitopes to be recognized and thus even the deleted mutant exu proteins would be efficiently detected.

2.3.1.2 Analysis of protein content by SDS Polyacrylamide Gel Electrophoresis (PAGE) and immunoblotting

The proteins were separated by SDS PAGE and then transferred onto nitrocellulose paper. The exu protein was probed with a rabbit anti-exu antibody and then a secondary antibody of alkaline phosphatase-conjugated anti-rabbit antibody. The color reaction was developed with NBT and BCIP. Details of the procedures of SDS PAGE and immunoblotting can be found in Appendix A.

2.3.2 Results

A western blot analysis of exu protein of ovary extracts of the transgenic flies is shown in Fig. 2.2. Ovary extracts from homozygous exu^{sc} were used as a negative control in which no exu protein was detected while ovary extracts from the w^{1118} served as a positive control. The wild type exu appeared as multiple bands due to post-translational modification at multiple sites by phosphorylation (Cheung et al., manuscript in preparation). Among the eight deletion transgenic constructs, exu protein bands were detected in six of them. All the bands were shown to have reductions in molecular weight corresponding to their respective deletions (Table 2.2). Only the rescue mutant, mutant Δ 442-503, consisted of an apparent doublet of exu protein while others appeared as single bands. This reflected that certain phosphorylation sites might have been retained in this mutant for postranslational modifications. Two transgenic constructs (mutants Δ 194-375 and Δ 390-531) had no detectable exu protein. One possible explanation for the lack of exu protein was the large deletions in these two constructs which rendered them unstable. Besides, it could also be due to the lack of *exu* mRNA which can be verified by RNase protection assay.

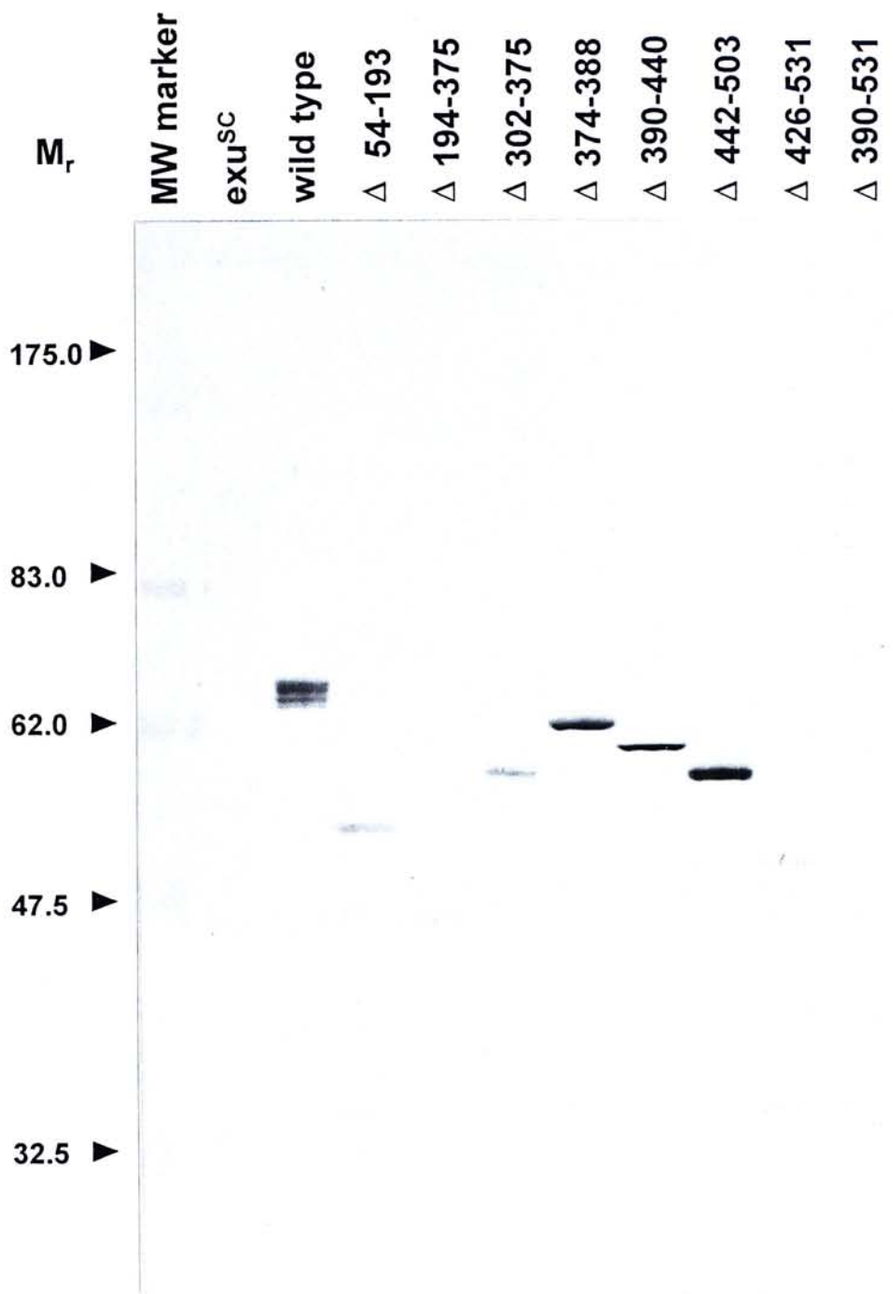


Figure 2.2 Analysis of exu protein in ovary extract of the deletion mutants.

The exu protein was detected by anti-exu antibody. Multiple isoforms of exu protein with different molecular weights can be detected in the wild type ovary extract. As a negative control, the ovary extract of the *exu^{sc}* protein null mutant was run in parallel with that of the wild type.

Lane: **exu^{sc}**, ovary extracts of the protein null mutant; **wild type**, ovary extracts of the wild type; Δ xxx-xxx, ovary extracts of the deletion mutants.

Table 2.2 The theoretical molecular weight and apparent relative molecular weight determined by SDS PAGE of exu proteins of the deletion mutants

Deletion mutants	theoretical M_r	apparent M_r
Δ54-193	43,000	41,500
Δ194-375	38,000	exu undetectable
Δ302-375	49,000	50,600
Δ374-388	56,000	55,500
Δ390-440	52,000	53,400
Δ442-503	51,000	50,000
Δ426-531	46,000	42,100
Δ390-531	42,000	exu undetectable

2.4 Localization of *bcd* mRNA and exu protein in oogenesis

2.4.1 Introduction

The spatial and temporal distribution of *bcd* mRNA is important for its localization to the anterior cortex of the oocyte. Four stages of *bcd* mRNA localization have been described by St. Johnston et al. (1989) and mutations of *exu*, *sww* and *stau* disrupt these stages of localization in a stepwise manner. Therefore, the localization seems to be proceeded in multiple steps in which *bcd* mRNA has to be localized correctly in each stage of oogenesis to achieve a tight localization in the embryo.

The colocalization of *bcd* mRNA and exu protein strongly suggests that they are associated during some stages of oogenesis. As their associations are only transient, exu protein would not involve in anchoring *bcd* mRNA by direct binding at the anterior pole of the oocyte. Rather, exu protein acts in initial steps of localization but not in maintenance of localization at late oogenesis and the embryo (Macdonald et al., 1991; Marcey et al., 1991).

Localization of several RNAs has been already known to depend on the preceding localization of other components. The posterior localization of *nanos*, cyclin B, and *gcl* mRNAs requires prior localization of *oskar* mRNA and oskar, staufen, and vasa proteins (Raff, et al., 1990, Jongens et al., 1992, Wang, et al., 1994; reviewed by St. Johnston, 1995). The preceding components may involve in binding to, or preparing binding sites for, the components which arrive later in oogenesis.

The spatial and temporal distribution of exu protein may be critical for localization of *bcd* mRNA. Localization of exu protein precedes that of *bcd* mRNA in the oocyte, and exu appears to concentrate in the oocyte at very early stages (Macdonald et al., 1991). Exu protein would thus have begun to function much earlier before *bcd* mRNA arrive at the oocyte. The function of the prior localization of exu would therefore be not directly involved in the binding of *bcd* mRNA but rather in preparing the localization machinery for *bcd* mRNA. Subsequent colocalization of exu protein and *bcd* mRNA at mid-oogenesis may then involve

either a direct or an indirect association of the two components which confers the tight anterior localization of *bcd* mRNA at late oogenesis. Thus, the localization of exu protein may play an essential role in the successful localization of *bcd* mRNA.

2.4.2 Spatial and temporal distribution of exu protein in the deletion mutants

To determine the significance of different regions of exu coding sequence on the spatial and temporal localization of exu protein, egg chambers were isolated from the deletion mutants and were analyzed by immunohistochemical staining using an anti-exu antibody.

2.4.2.1 Materials and Methods

The immunohistochemical staining protocol was essentially as described by Macdonald et al. (1991) with modifications. About 20 pairs of ovaries were dissected from well-fed two-day-old female flies in PBS (8g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L of double-distilled water, adjusted to pH 7.4 with HCl). The ovaries were briefly washed in PBS in a microfuge tube. Individual ovarioles were dispersed by repeatedly pipeting the ovaries in a 50 µl volume of PBS.

To allow better penetration by antibodies, egg chambers were incubated in 0.375M KCl at 37°C for 30 minutes. The egg chambers were washed twice in PBS and then fixed in 4% formaldehyde in PEM buffer (0.1 M PIPES, pH 6.8, 2 mM $MgSO_4$, 1 mM EGTA) for 17 minutes at room temperature with rotation. The egg chambers were briefly washed twice in PTW (PBS, 0.1 % Tween 20), twice for 30 minutes in PTW, and then incubated with a rabbit anti-exu antibody diluted 1:800 in TNBTT (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 % BSA, 0.1 % Triton X-100, 0.05 % thimerosal). After incubation overnight at 4°C with rotation, the egg chambers were washed twice in TNBTT and then blocked with 2% normal goat serum (Antibodies Incorporated) in TNBTT for 30 minutes at room temperature. After washing twice for 30 minutes in TNBTT, the egg chambers were further incubated with an FITC-conjugated goat anti-rabbit secondary antibody (Vector Laboratories) in 1:1200 dilution in TNBTT containing 1 % BSA for two hours at room temperature. The egg chambers were washed three times for 15 minutes in PTW and then mounted in fluorescent label mounting medium (0.1 M Tris-HCl, pH

8.0, 90 % glycerol, 2.3 % of 1,4-diazobicyclo-(2,2,2)-octane (DABCO) (w/v), 0.02 % sodium azide), and examined with a Zeiss epifluorescent microscope.

2.4.2.2 Results

Distribution of exu in wild type ovaries

The spatial and temporal distribution of exu protein was essentially as those described previously (Marcey et al., 1991; Macdonald et al., 1991; Wang and Hazerigg, 1994). In the wild type ovaries, exu protein was expressed in the germarium as well as in the oocytes of early stages egg chambers (Figure 2.3A). The exu protein was observed to be localized exclusively in the oocytes from stage 3 to stage 8 (stages according to King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993). Beginning from stage 8, exu protein was localized in the nurse cells but most of the exu still remained in the oocyte. At stage 9, exu protein continued to accumulate in the apical regions of the nurse cells and there was a concomitant reduction of exu protein in the oocyte, where little or no exu protein accumulated in stage 10A.

Distribution of exu in *exu*^{sc} mutant

As the *exu*^{sc} allele is a protein null mutation, essentially background staining was observed at all stages of egg chambers as expected (Figure 2.3A). The egg chambers exhibited a homogenous faint background staining indicating no detectable exu protein.

Distribution of exu in deletion mutants

Several groups of pattern of the spatial and temporal distribution of exu protein were observed in the deletion mutants. One of the deletion mutants exhibited very similar distribution of exu protein as that of the wild type. This mutant, Δ 442-503 had exu protein localized in early oocytes and in the nurse cells at late stages (Figure 2.3A). It was noteworthy that this mutant carried a transgene that could rescue the maternal-effect lethality and had the same distribution pattern of exu protein as the wild type. Thus, this transgene was functionally indistinguishable from that of the wild type *exu* although it had 62 internal amino acid residues deleted (see section 2.2.2).

The second group had distribution pattern resembling only some of the localization pattern of the wild type. In two of the mutants, Δ302-375 and Δ374-388 (Figure 2.3B), their distribution of exu protein differed from the wild type only in the loss of localization at the early stages oocytes (from germarium up to stage 5), where little or no exu protein was detected. The absence of localization would either be due to the failure of exu to be localized or to the lack of expression in the early stages. Although localization was observed in the oocytes of the stages 6 to 9 egg chambers, the level of exu protein was much lower than that of the wild type. In addition, exu protein was concentrated in stage 10A nurse cells and localized at the apical regions as in the wild type.

The third group had one part of the distribution pattern similar to that of the second group in which no localization of exu was observed from germarium to stage 5 and low level of localization was observed in oocytes between stages 6 to 9 egg chambers. However, in mutants Δ390-440 and Δ426-531 (Figure 2.3C), the level of exu protein concentrated in the nurse cells was much lower than that of the second group in stage 10A egg chambers. Western blot analysis had confirmed that the level of exu protein in these two mutants was lower than that of the mutants of the second group. Therefore, the absence of concentration of exu in stage 10A nurse cells of mutants Δ390-440 and Δ426-531 might be due to the low level of expression, and thus the mutants from both the second and the third groups were represented similar distribution pattern of exu at early stages. None of these transgenes was able to rescue the maternal-effect lethality and hence the distribution patterns of the mutated exu protein appeared to correlate with their loss of function.

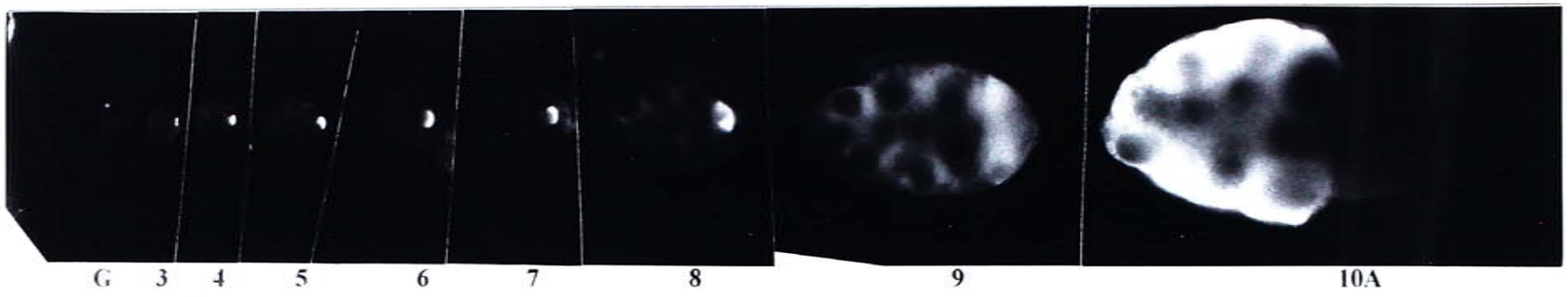
In the fourth group of distribution pattern, mutant Δ54-193 (Figure 2.3D) had no localization of exu protein at very early egg chambers up to stage 9. As exu protein bands were detected by western blot analysis, the absence of localization was due to the inability of the exu protein to get localized which resulted in a homogenous distribution of the protein. Alternatively, the expression of exu was lower in the early stages but began to accumulate in stage 10A egg chambers, where low level of exu protein was detected in the nurse cells.

In the fifth kind of distribution pattern, egg chambers of mutants Δ194-375 and Δ390-531 (Figure 2.3E), had no detectable exu protein. The phenotype of these

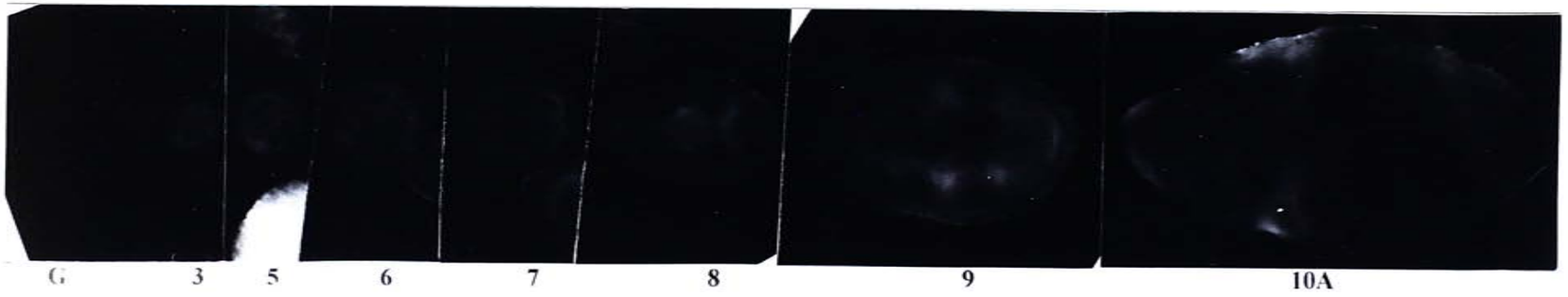
two mutants was essentially the same as that of the protein null mutant *exu*^{sc}. This observation was confirmed by western blot analysis in which little or no exu protein was detected. Therefore the absence of exu localization was due to lack of exu protein expressed or instability of mutant exu mRNA and protein in these mutants.

Figure 2.3A Spatial and temporal expression of exu protein of the deletion mutants during oogenesis. Based on the pattern of distribution of exu protein, the eight deletion mutants were divided into five groups. The wild type, *exu*^{sc} (protein null mutation), and the mutant, Δ442-503, of the first group of exu protein distribution pattern, which was very similar to that of the wild type.

w^{1118}
(wild type)



exu^{sc}



mutant
Δ442-503

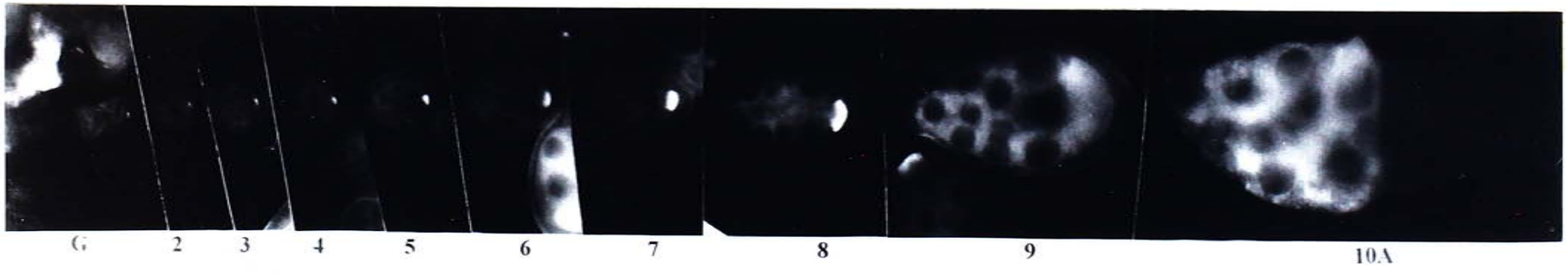
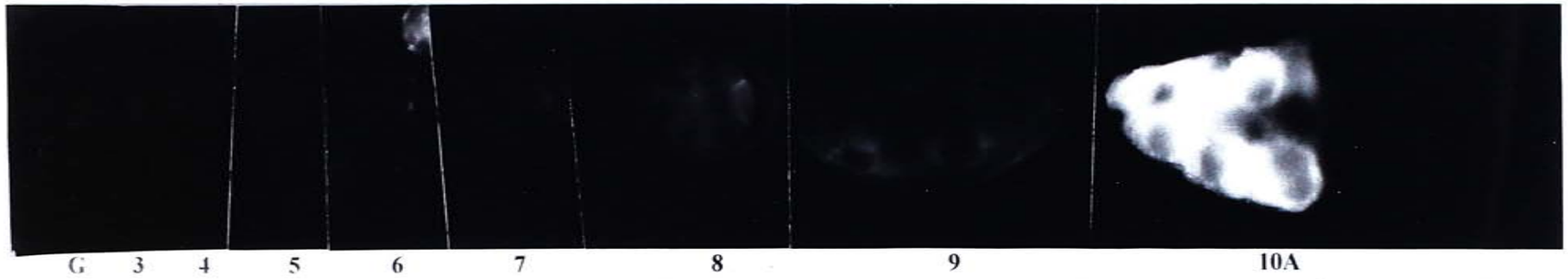


Figure 2.3B The second group of exu protein distribution pattern. In mutants Δ302-375 and Δ374-388, localization of exu was lost from stages 1 to 5 while it was partially retained in mid-oogenesis and was indistinguishable from the wild type in stages 9 and 10A.

mutant
Δ302-375



mutant
Δ374-388

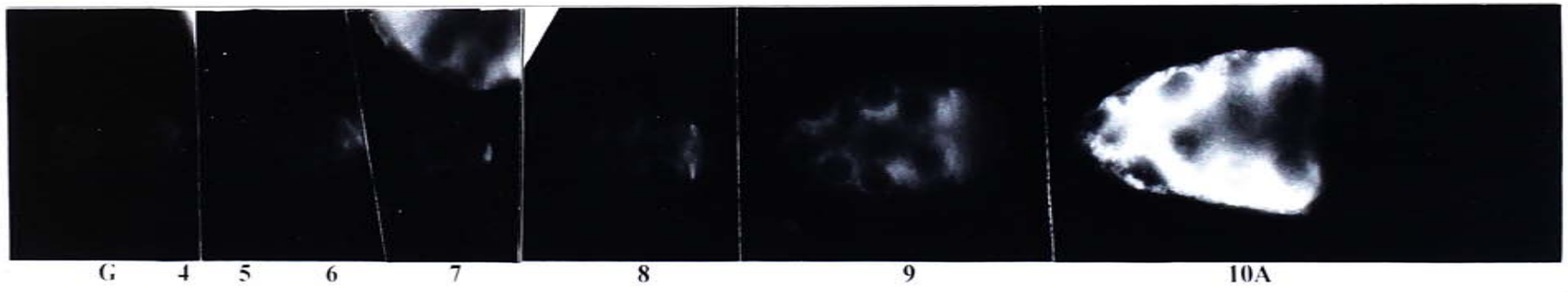
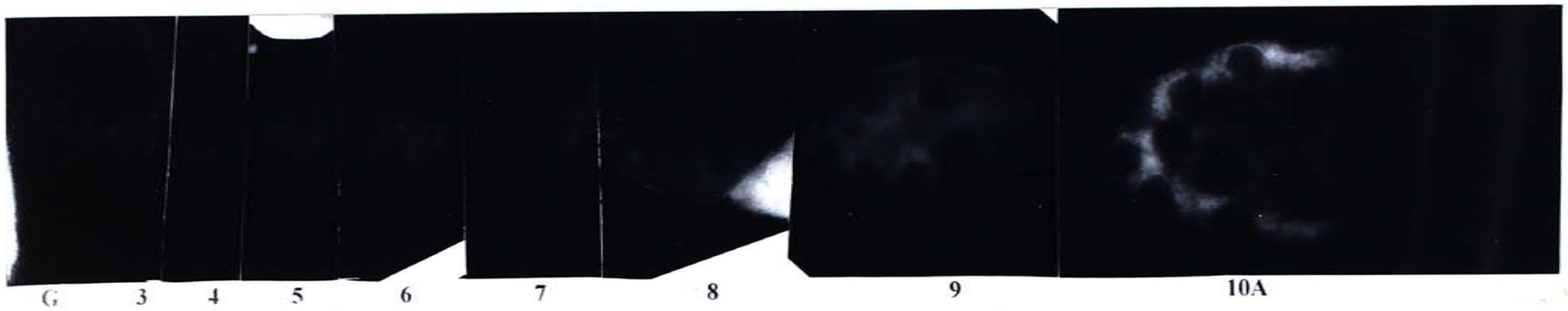


Figure 2.3C The third group of exu protein distribution pattern. In mutants Δ390-440 and Δ426-531, localization of exu in early stages (1-5) and late stages (9-10A) was not observed but was partially retained at some stages in mid-oogenesis (6-8).

mutant

Δ390-440



mutant
Δ426-531

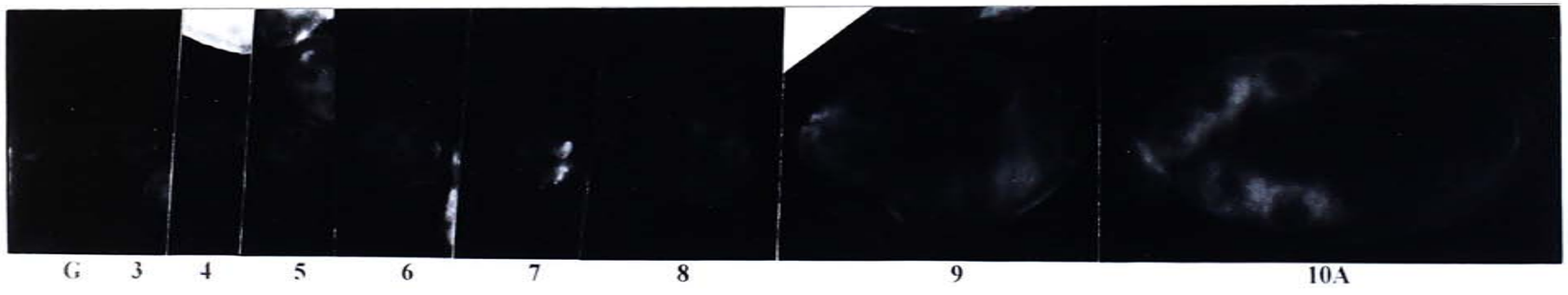


Figure 2.3D The fourth group of exu protein distribution pattern. Only one mutant, Δ54-193 exhibited no localization of exu in all stages examined.

mutant
Δ54-193

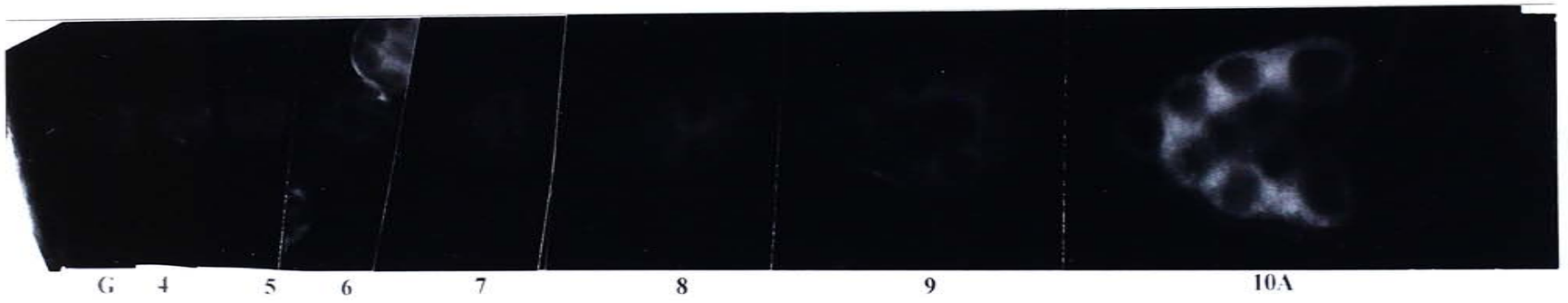
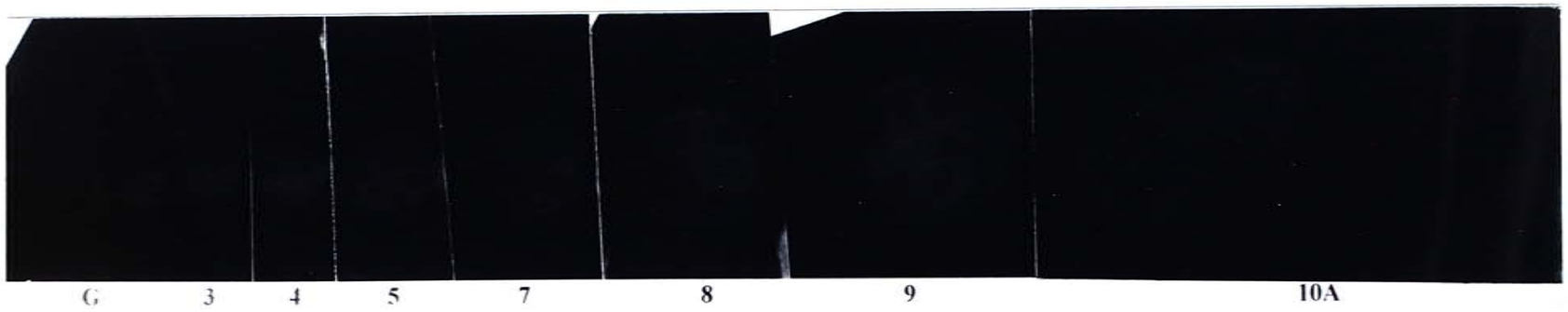


Figure 2.3E The fifth group of exu protein distribution pattern. In mutants Δ194-375 and Δ390-531, no exu protein was detected. The observation agreed with the absence of exu protein detected by western blot.

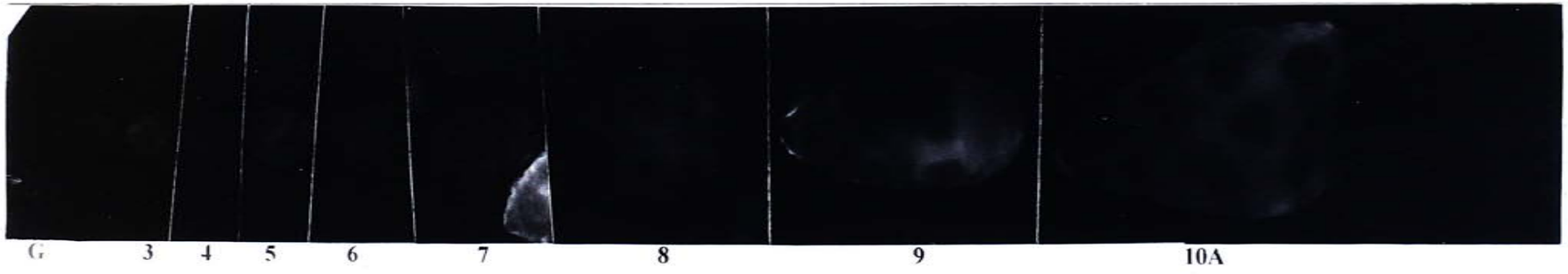
mutant

Δ194-375



mutant

Δ390-531



2.4.3 Spatial and temporal distribution of *bcd* mRNA in the deletion mutants

A function assay of the exu deletion mutants was performed by whole mount *in situ* hybridization to analyze the spatial and temporal distribution of *bcd* mRNA. Failure of proper localization of *bcd* mRNA represented loss of function of the *exu* and thus the functional significance of the deleted coding sequences could be examined.

2.4.3.1 Materials and Methods

2.4.3.1.1 Principles of DIG-labeling and *in situ* hybridization

The labeling reaction was performed using the DIG DNA labeling and detection kit (Boehringer) with modifications as described by Macdonald et al. (1991). DNA was labeled by random primed incorporation of digoxigenin (DIG)-labeled deoxyuridine-triphosphate (dUTP). A steroid hapten digoxigenin was linked to the dUTP through a spacer arm. This DIG hapten allowed easy recognition of the target hybrids after hybridization.

The template double-stranded DNA was first linearized and then denatured by heating. Random hexanucleotides that bind to the single-stranded DNA were used as primers for DNA replication in the presence of dNTP and DIG-conjugated dUTP (Figure 2.4A). The egg chambers were fixed and washed, and then probed with the DIG-labeled DNA as described in section 2.4.3.1.3 (Figure 2.4B). After hybridization, the hybrids were detected by enzyme-linked immunoassay using anti-DIG-alkaline phosphatase conjugate and subsequent enzyme-catalyzed colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitro blue tetrazolium salt (NBT).

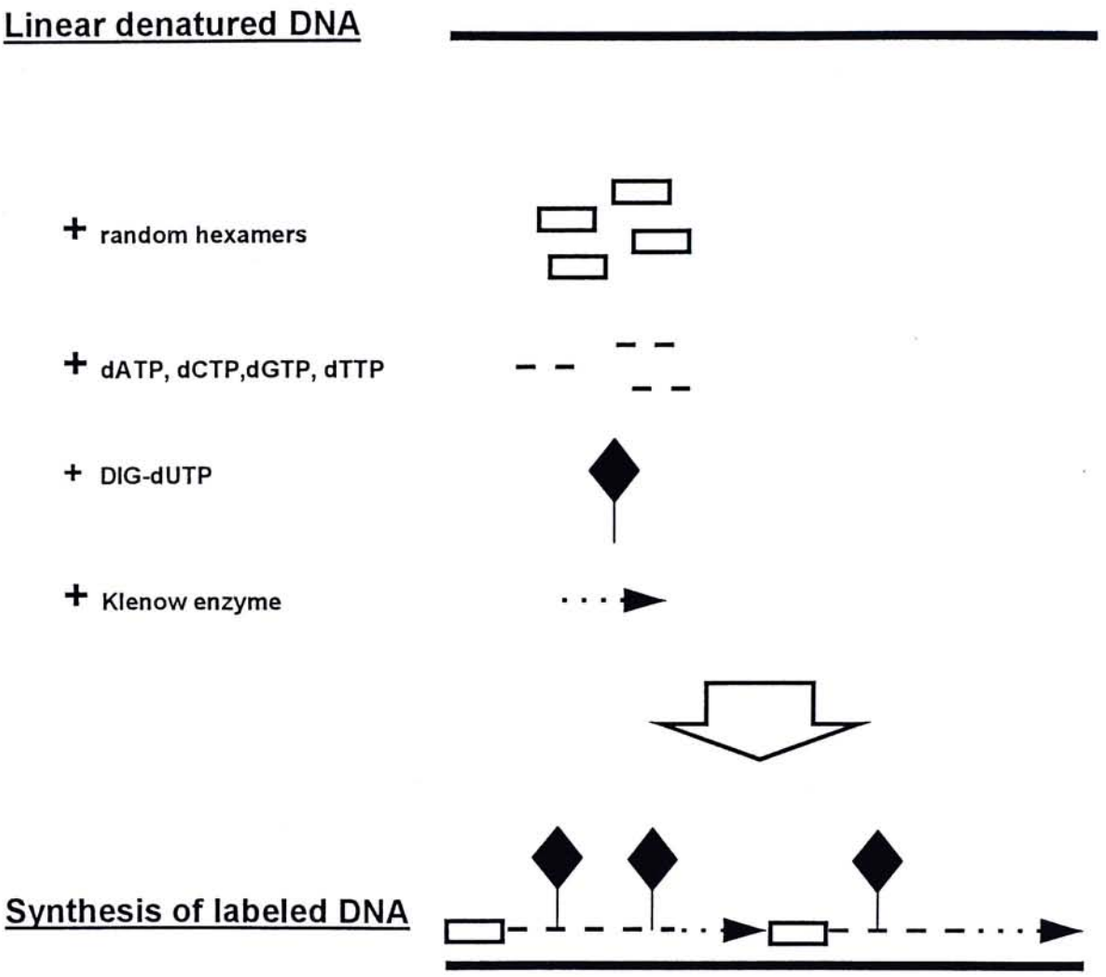


Figure 2.4A Synthesis of DIG labeled DNA probe by random priming. (Adapted from the menu of the DIG DNA Labeling and Detection Kit Nonradioactive, Boehringer)

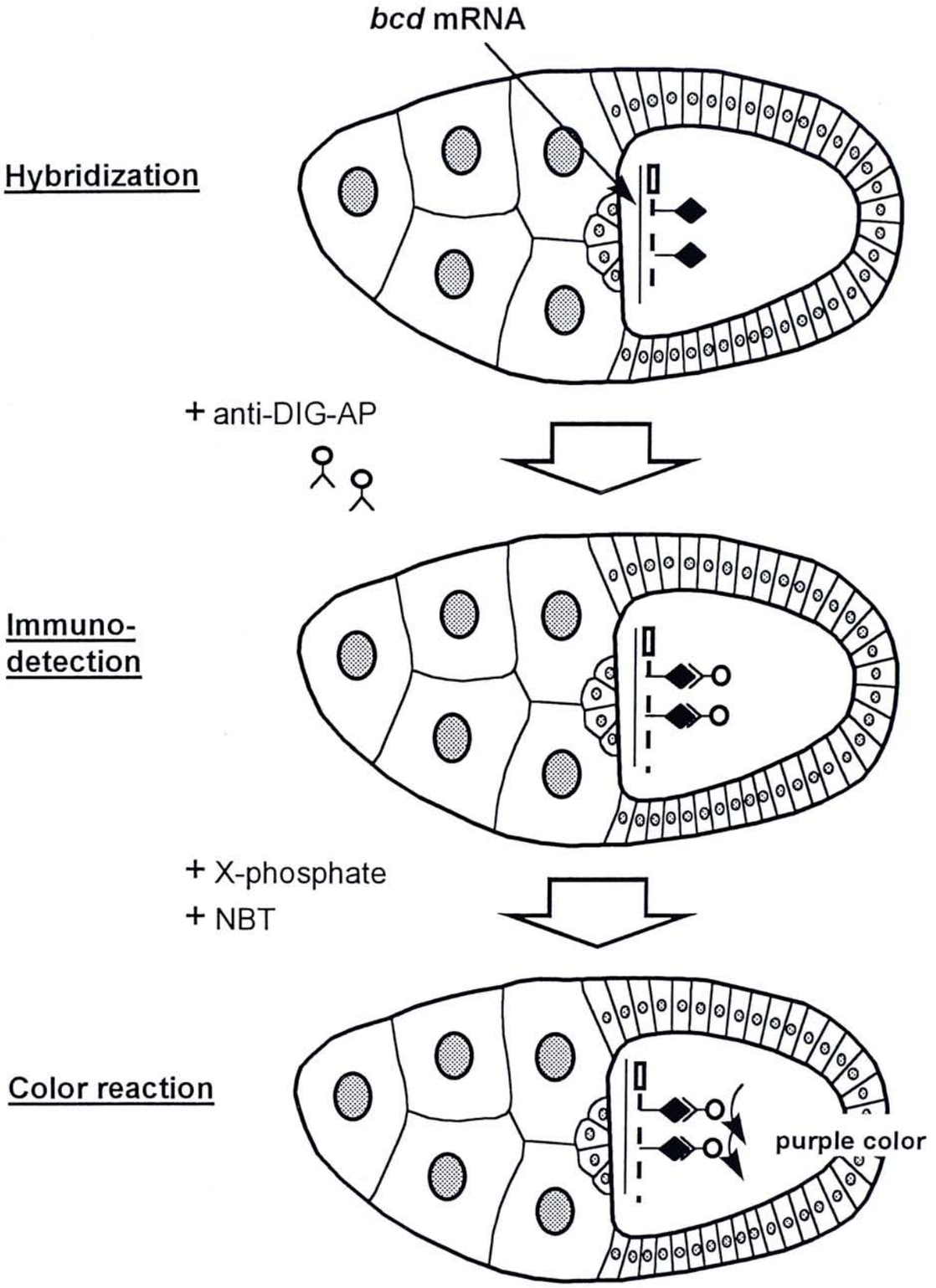


Figure 2.4B Diagrammatic representation of *in situ* hybridization of egg chambers using the DIG labeled DNA probe.

2.4.3.1.2 Synthesis of DIG-labeled *bcd* DNA probe

A 1.7 kb EcoRI / RV fragment was released from a plasmid p1122, which contained the *bcd* cDNA clone, for synthesis of DIG-labeled probe. Details of the procedures for preparation of DNA template can be found in Appendix A.

DIG-labeled DNA probe was made by random primed labeling on the DNA template using a Boehringer Mannheim genius kit (DIG DNA Labeling and Detection Kit, Non-radioactive). The purified RI / RV fragment was resuspended in 58 μl double distilled water to make a final volume of 100 μl. The tube was boiled for 10 minutes and then quickly chilled on a dry ice / ethanol bath. The frozen DNA was allowed to thaw at room temperature. Before all the DNA solution was thawed, the following were added in sequence.

(1) 10X LB 10 μl

(LB: 50 mM HEPES, pH 6.9, 5 mM $MgCl_2$, 2 mM DTT)

(2) 10X hexamer 10 μl

(3) 10X dNTP 10 μl

(4) Klenow (2 U/μl) 2 μl

The content was mixed and incubated overnight at 37°C. The labeled DNA was ethanol-precipitated and resuspended in 1 ml hybridization solution. The probe was kept at -20°C before use.

The efficiency of the labeling reaction was tested by immobilizing the probe on nitrocellulose paper, detecting with an anti-DIG antibody and NBT / X-phosphate color reaction. Details of probe testing can be found in Appendix A.

2.4.3.1.3 *in situ* hybridization of *bcd* mRNA in egg chambers using DIG-labeled DNA probe

The *in situ* hybridization was based on the protocol described by Macdonald (1992) with modifications. About 20 pairs of ovaries were dissected from well-fed two-day-old female flies in PBS. The ovaries were dispersed in the same way as in the immunohistochemical staining.

The egg chambers were incubated in 0.75 M KCl in PBS at 37°C for 30 minutes. The KCl solution was removed and the egg chambers were rinsed twice in PBS. The egg chambers were fixed in 4 % paraformaldehyde in PBS for 20 to 30

minutes with rotation (usually by rotating the microfuge on a motor-driven rotator) and then were rinsed three times for 5 minutes in PTW. In order to enhance probe penetration, the egg chambers were digested in 50 ug/ml of non-digested proteinase K (Boehringer) in PTW for 8 to 9 minutes. The proteinase K digestion was stopped by incubation in 2 mg/ml of glycine in PTW for 2 minutes. The egg chambers were rinsed three times for 5 minutes in PTW and then refixed in 4 % paraformaldehyde in PBS for 20 minutes.

The egg chambers were washed at least five times for 5 minutes in PTW, followed by a wash in 1:1 mixture of PTW and hybridization solution (50 % formamide, 5X SSC, 100 ug/ml sonicated salmon sperm DNA, 50 ug/ml heparin, 0.1 % Tween 20) for 10 minutes and a wash in hybridization solution for 10 minutes. About 0.5 ml of hybridization solution was heated in a boiling water bath for 5 minutes and then chilled on ice. The egg chambers were prehybridized in this solution for one hour at 50°C and then hybridized with 100-150 µl of denatured DIG-labeled DNA probein hybridization solution overnight at 50°C

At the end of the hybridization, the egg chambers were washed for 20 minutes in hybridization solution at 50°C. A series of washes were done sequentially in the following solutions for 20 minutes at 50°C.

(1) 4 parts of hybridization solution and 1 part of PTW

(2) 3 parts of hybridization solution and 2 parts of PTW

(3) 2 parts of hybridization solution and 3 parts of PTW

(4) 1 part of hybridization solution and 4 parts of PTW

The egg chambers were further washed twice for 20 minutes in PTW at room temperature . All subsequent steps were done at room temperature.

Non-specific binding sites of the egg chambers were blocked twice for 20 minutes using PBT (PBS, 0.1 % BSA, 0.2 % Triton X-100). A 1:5000 dilution of alkaline phosphatase (AP)-conjugated goat anti-DIG antibody in PBT was added. After incubation for one hour, the egg chambers were rinsed four times for 20 minutes in PBT. Before staining, the egg chambers were washed three times for 5 minutes in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). NBT(0.45 % v/v; 0.34 mg /ml) and X-phosphate(0.3 % v/v; 0.15 mg /ml) (both supplied with the kit) in AP buffer were used for the color development of the AP

reaction. The optimal time for staining was approximately 90 minutes. The staining reaction was stopped by washing the egg chambers several times in PBS and the egg chambers were dehydrated by quickly rinsing through a series of ethanol solutions (30%, 50%, 70% and 100%) before mounting onto slides using GMM (Canada Balsam (Sigma) mixed with methyl salicylate in a ratio of 17 : 1 (v/v)).

2.4.3.2 Results

Distribution of *bcd* mRNA in wild type

The spatial and temporal distribution of *bcd* mRNA was essentially the same as those described by St. Johnston et al. (1989). In the wild type ovaries, *bcd* mRNA first appeared at stage 6 in which it was accumulated at the oocyte (Figure 2.5A). Through stage 7 to 9, *bcd* mRNA was confined in the anterior margin of the oocyte. At stage 9 and 10A, localization of *bcd* mRNA was observed both at the anterior margin of the oocyte and at the apical regions of the nurse cells. The localization was tightly restricted to the anterior margin at stage 10A oocyte.

Distribution of *bcd* mRNA in exu^{sc} mutant

In the exu^{sc} mutant, distribution of *bcd* mRNA seemed to be similar to the wild type from stages 6 to 8. Beginning from stage 9, *bcd* mRNA was localized at the anterior margin of the oocyte but the localization appeared as diffused (Figure 2.5A). At stage 10A, a critical difference was observed that *bcd* mRNA was not localized to the anterior margin but rather formed a gradient that diffused from the anterior to the posterior within the oocyte. In addition, *bcd* mRNA was distributed evenly in the nurse cells where apical localization was not observed.

Distribution of *bcd* mRNA in deletion mutants

In the rescue mutant, Δ442-503, localization of *bcd* mRNA was comparable to that of the wild type. The *bcd* mRNA first appeared in the oocyte at stage 6 and was confined at the anterior margin from stage 7 through stage 9 (Figure 2.5A). At stage 10A, it was tightly localized at the anterior margin of the oocyte and was also localized in apical regions of the nurse cells.

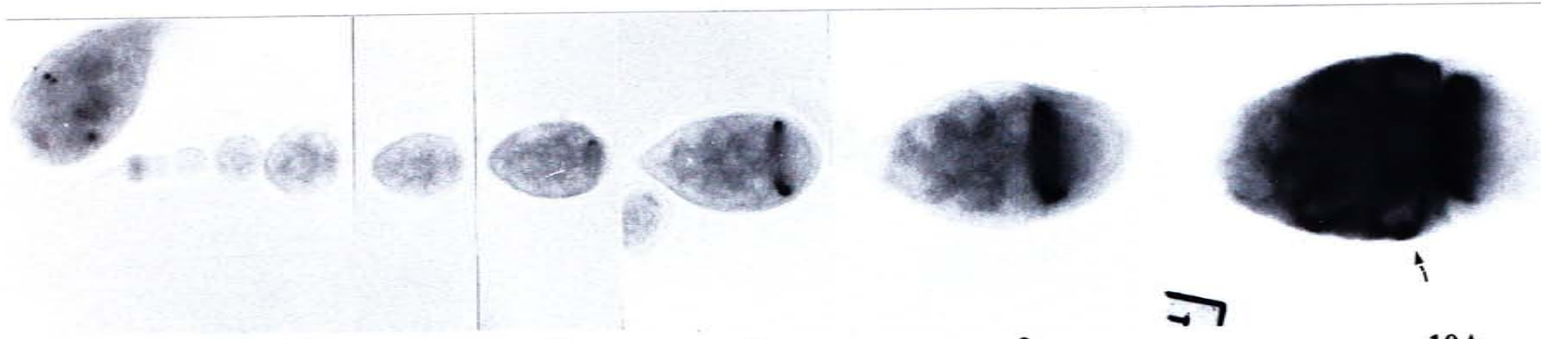
For all other deletion mutants, *bcd* mRNA was not tightly localized at the anterior end of the oocyte. Based on the distribution pattern of *bcd* mRNA, these mutants could be divided into two groups. In mutants Δ302-375, Δ374-388, Δ390-440 and Δ426-531, low level of *bcd* mRNA was localized in the oocytes from stages 6 to 8 (Figure 2.5B). However, *bcd* mRNA was localized at the anterior end of the stage 9 oocyte but appeared to be much more diffused in comparison with

the wild type. At stage 10A, the distribution of *bcd* mRNA was uniform within the oocyte but a higher concentration of *bcd* mRNA was observed at the anterior end. In the nurse cells, localization of *bcd* mRNA at the apical regions of the nurse cells was observed but the level of localization was much lower than that in the wild type, and most of *bcd* mRNA remained evenly distributed in the nurse cells.

The third group of distribution pattern included mutants Δ54-193, Δ194-375 and Δ390-531, which showed very similar distribution patterns of *bcd* mRNA. From stages 6 to 8, little localization of *bcd* mRNA was observed in the oocytes (Figure 2.5C). At stages 9 and 10A, uniform distribution of *bcd* mRNA was observed in the oocyte as well as in the nurse cells, and higher concentration at the anterior end of the oocyte was also observed in stage 10A.

Figure 2.5A Spatial and temporal expression of *bcd* mRNA during oogenesis. Three main groups of pattern of distribution of *bcd* mRNA were observed among the eight deletion mutants. The wild type, exu^{sc} (protein null mutation), and the mutant Δ442-503 of the first group of pattern which had similar localization of *bcd* mRNA to that of the wild type. Arrows indicate the apical localization in the nurse cells.

wild type



exu^{sc}

mutant
Δ442-503

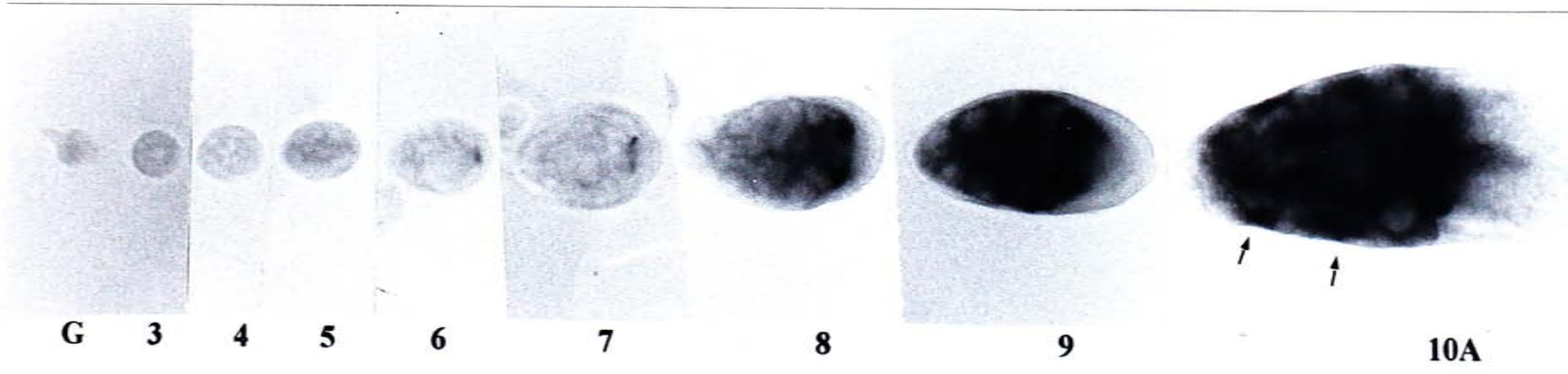


Figure 2.5B The second group of *bcd* mRNA distribution pattern. Four mutants, Δ302-375, Δ374-388, Δ390-440 and Δ426-531, showed diffused anterior localization of *bcd* mRNA in oocytes of stage 9 egg chamber, and partial localization at the anterior end of the oocyte and apical regions (indicated by arrows) of the nurse cells of the stage 10A egg chamber.

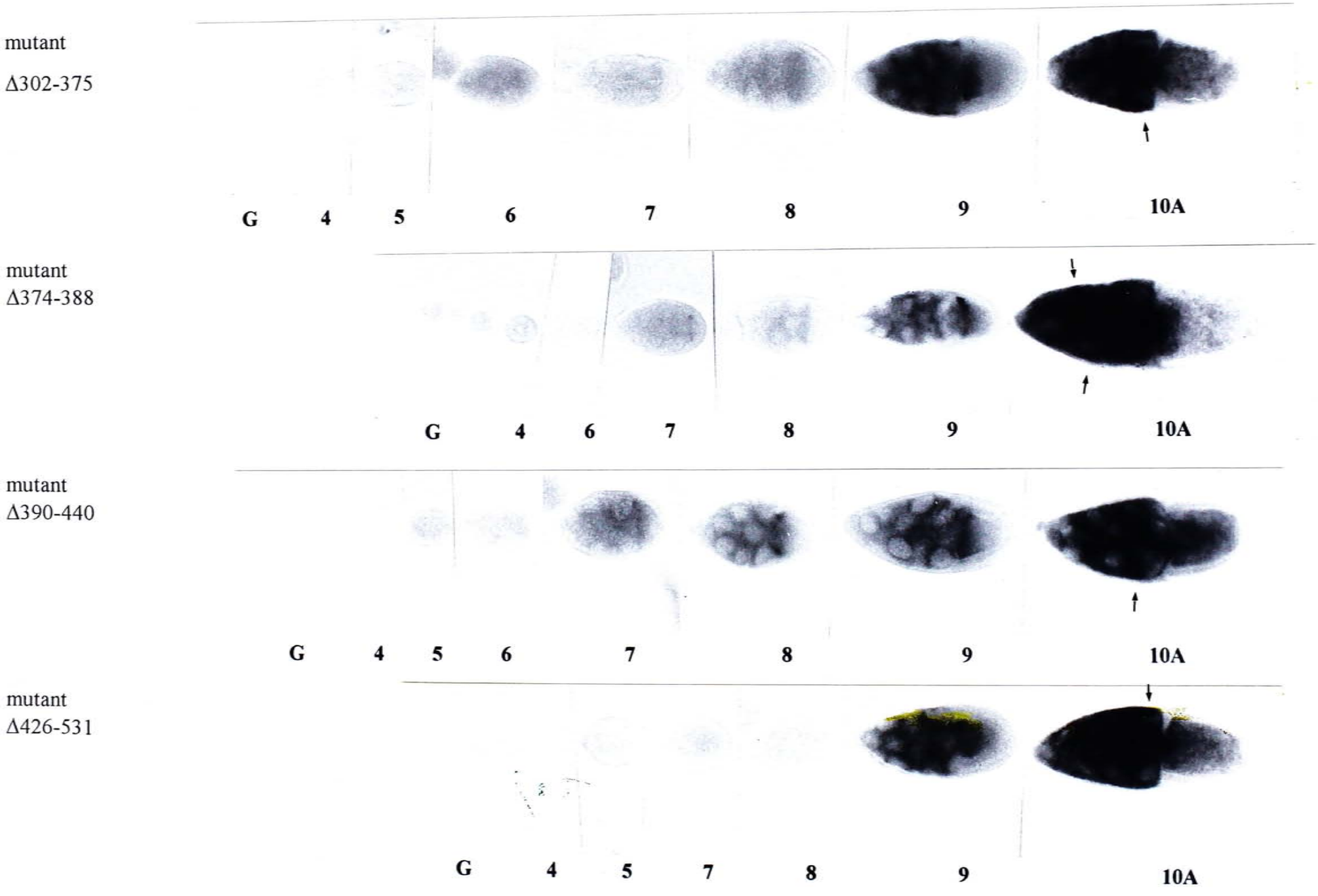
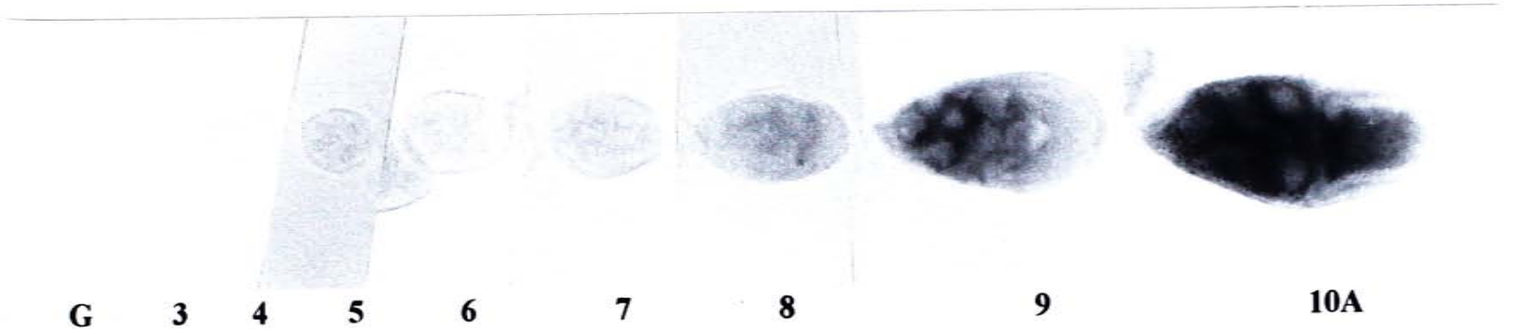
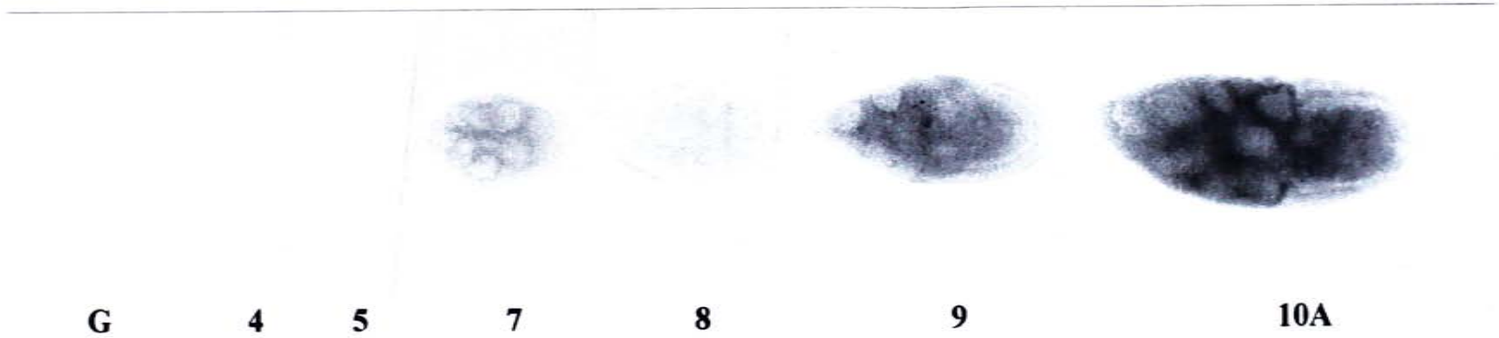

mutant
Δ302-375
G 4 5 6 7 8 9 10A
mutant
Δ374-388
G 4 6 7 8 9 10A
mutant
Δ390-440
G 4 5 6 7 8 9 10A
mutant
Δ426-531
G 4 5 7 8 9 10A

Figure 2.5C The third group of *bcd* mRNA distribution pattern included three mutants. Three mutants Δ54-193, Δ194-375 and Δ390-531 exhibited a uniform distribution of *bcd* mRNA in stages 9 to 10A egg chambers and higher concentration at the anterior end of stage 10A oocyte.

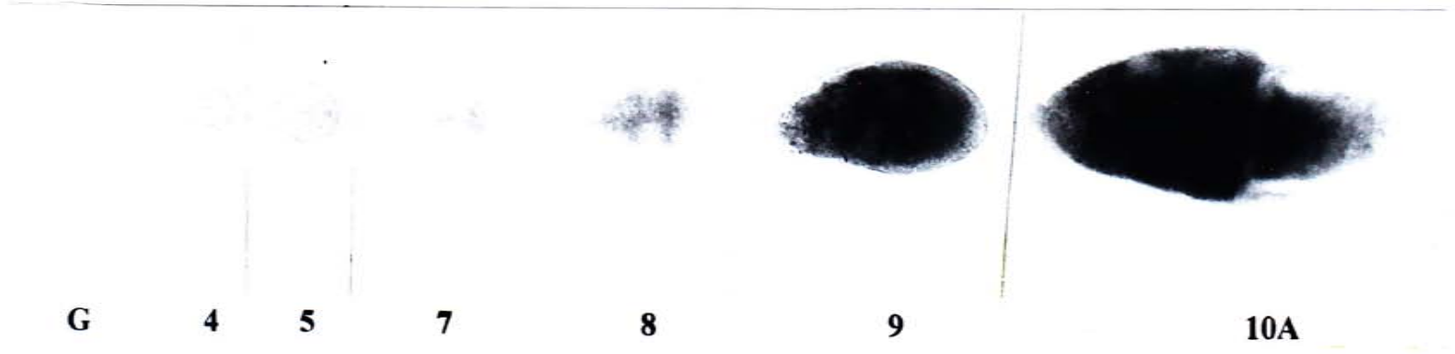
mutant
Δ54-193



mutant
Δ194-375



mutant
Δ390-531



2.5 Discussion

Deletion analysis of the exu protein has demonstrated that the deletion of 62 amino acid residues in mutant Δ442-503 was dispensable for function. This mutant could provide all the *in vivo* functions of the wild type *exu*. In the rescue assay, the mutant Δ442-503 could rescue the maternal-effect lethality as well as the sterility of homozygous exu^{sc}. By immunohistochemical staining and *in situ* hybridization, this mutant exhibited the same spatial and temporal distribution of both exu protein and *bcd* mRNA as those of the wild type during oogenesis. Thus this mutant was indistinguishable from the wild type with respect to both phenotype and function.

For the other seven deletion mutants, none of them could rescue the maternal-effect lethality of homozygous exu^{sc}. Therefore, they did not complement the protein null exu^{sc} allele in providing the *in vivo* functions of the wild type *exu*. Theoretically, loss-of-function mutations reflect the presence of functionally important regions within the deleted protein sequence. Thus, almost the entire coding sequence, except for the 62 amino acid residues deleted in mutant Δ442-503, seemed to be functionally important. However, the loss of function of the deletion mutants might not be totally due to the removal of functional domain(s) but could involve domains not directly responsible for function. Domains responsible for maintaining the correct conformation of the functional domain(s) were perhaps disrupted in the deletion mutants. Alternatively, stability of the protein was also possibly affected. The PEST domain, which was speculated to confer rapid proteolysis, was found in the C-terminal region of exu protein. The PEST sequence was removed or partially deleted in four of the deletion mutants (Table 2.3) and the stability of these mutant exu proteins might have been altered. Nonetheless, the rescue mutant Δ442-503 had over half of the PEST sequence removed. Therefore, the PEST domain seemed to be not necessarily important for the function of exu protein. Furthermore, it is noteworthy that one of the non-rescue mutants, Δ374-388 had a deleted region consisting of only five amino acid residues (3 % of the full length exu). These amino acid residues might account for important function or provide essential structural information necessary for the maintenance of the functional domains.

The non-rescue mutants exhibited altered patterns of spatial and temporal distribution of both exu protein and *bcd* mRNA. All of them had lost the tight localization of *bcd* mRNA both at the anterior end of the oocyte and at the apical region of the nurse cells in stage 10A egg chambers, suggesting that the absence of rescue activity in these mutants was due to the defects in *bcd* mRNA localization. Based on the distribution pattern of *bcd* mRNA, the non-rescue deletion mutants could be divided into two groups. One group of mutants, Δ302-375, Δ374-388, Δ390-440 and Δ426-531 had localization of *bcd* mRNA at the anterior end of the oocyte but the localization was quite diffused in stage 9 egg chambers. At stage 10A oocytes, *bcd* mRNA was distributed homogeneously within the oocyte and the nurse cells, despite higher levels of *bcd* mRNA were present at the anterior margin of the oocyte and the apical regions in the nurse cells. This partial localization of *bcd* mRNA suggested that some of the functions of exu protein might be retained in these mutants. On the other hand, the distribution pattern of exu protein in these four mutants were not quite similar, at least at the late stages (10A). In mutants, Δ302-375 and Δ374-388, localization of exu protein in the oocytes was observed between stages 6-8, although the level of localization was much lower than that of the wild type. Beginning at stage 9, localization of exu protein in the apical regions of the nurse cells was observed and exu protein was almost exclusively found in the nurse cells at stage 10A egg chambers (Table 2.4). The other two mutants, Δ390-440 and Δ426-531, also had localization of exu protein in the oocytes between stages 6-8 but no apical localization in the nurse cells of the stages 9 and 10A egg chambers (Table 2.4). Besides, the level of exu protein in the stages 10A egg chambers was much lower than that of mutants , Δ302-375 and Δ374-388.

In the other three non-rescue mutants, Δ54-193, Δ194-375 and Δ390-531, *bcd* mRNA was uniformly distributed but a higher level of *bcd* mRNA was found at the anterior end of the oocyte in stage 10A egg chambers. The failure of localization would be due to the absence of localization of exu protein in mutant Δ54-193 and to the stability of the protein in the other two mutants . The large sequence deletions in these three mutants might have led to instability of the mutant protein. The deleted regions accounted for more than 25 % of the full length protein in the three mutants (Table 2.5). Low level of expression in mutant Δ54-193 and

even absence of detectable exu protein in mutants Δ194-375 and Δ390-531 supported the fact that the protein expression was greatly affected. The large deletions in coding sequence would possibly affect the expression in both the transcriptional and translational levels. The resulting mRNA and polypeptides would lack essential structural elements for maintaining their structure and stability and thus no protein could be accumulated to the detectable level.

By comparison of the spatial and temporal distribution of exu protein and *bcd* mRNA among the eight deletion mutants (Table 2.4), it was obvious that the non-rescue mutants differed from the rescue mutant in loss of localization of exu protein at early stages (stages 1-5) and failure of localization of *bcd* mRNA at late stages. Thus exu protein of the non-rescue mutants might have lost certain functional domains necessary for its localization at the early stages, and this early localization was critical for subsequent localization of *bcd* mRNA at late stages. Therefore, the localization of exu protein at early stages would play a significant role in *bcd* mRNA localization.

In addition, among the deletion mutants, the localization of exu protein can be distinguished into three phases. The first phase was the localization of exu protein in the oocytes at stages 1-5, which was only observed in the rescue mutant (Δ 442-503). The second phase was the localization in the oocytes at the mid-oogenesis (stages 6-8) and the third phase was found at stages 9 and 10A in which exu protein was gradually excluded from the oocyte and concentrated in the apical regions of the nurse cells. The different phases of exu localization reflects the presence of different potential functional domains regulating its localization at different stages. Three different potential domains of exu protein may be responsible for directing its localization at early stages (stages 1 to 5), mid-oogenesis (stages 6 to 8) and late stages (stages 9-10A). Alternatively, the three domains may work cooperatively as a single functional unit to direct the exu localization. The localization directed by these potential domains in turn confers *bcd* mRNA localization at different stages. In the rescue mutant, all three domains must have been retained and thus anterior localization of *bcd* mRNA was observed at both stage 9 and stage 10A (Table 2.4). In the non-rescue mutants (Δ 302-375, Δ374-388, Δ390-440 and Δ426-531), the second domain might have been preserved as

anterior *bcd* mRNA localization was only found in stage 9 but not in stage 10A (Table 2.4). Thus the potential functional domains may confer *bcd* mRNA localization in a stepwise manner - the first domain is responsible for the localization of stage 10A while the second domain is sufficient to confer localization only at stage 9.

Based on the assumption that different domains of exu direct localization of *bcd* mRNA at different stages, several regions of the coding sequence of *exu* seemed to confer different functions (Figure 2.6). A region starting from amino acid residues 54 to 193 would be important for the localization of *bcd* mRNA in both stages 9 and 10A, as removal of this region resulted in loss of the localization. The other two regions, amino acid residues 302-442 and 503-531, would be responsible for conferring localization in stage 10A, since mutants having deletions within these two regions lost their localization of *bcd* mRNA in stage 10A but not in stage 9. Thus all the three regions appeared to be essential for the *bcd* mRNA localization. In addition, deletion of two regions which spanned amino acid residues 194-375 and 390-531 resulted in absence of detectable exu protein. These regions would be necessary for maintaining the stability of exu protein. Alternatively, the regions affecting the *bcd* localization might be also important for the stability of the protein, as lower level of protein was detected in some of the mutants (Δ54-193, Δ390-440 and Δ426-531). However, this model may be oversimplified as the functional domains of exu are likely to exist in three-dimensional conformation. Tertiary structure of the protein may bring different regions of the coding sequence into a specialized conformation. Thus the three different regions conferring the *bcd* mRNA localization might represent different parts of a single functional unit and all of them may be required for the full activity of exu.

The experimental results suggested a possible mechanism of how exu protein assists in localization of *bcd* mRNA. As the localization of exu protein at the early stage oocytes seems to be important functionally, certain localization signals may be deposited in the early oocytes by exu protein to direct the localization of *bcd* mRNA at late stages. Direct interactions between exu protein and *bcd* mRNA are not necessary in this model. Rather, additional components may be essential for their indirect linkage in which they receive signals from exu protein

and then transmit the signals to other components necessary for the localization. In this model, two unknown components have not been identified yet. The first is the one which initiates exu protein to transmit the localization signals and the second is the one which receives the signals from exu protein. In combination to the model proposed by Lane and Kalderon (1994), the first component is thought to be resided within the posterior polar follicle cells, whose position readily provides an asymmetry to the oocyte. A signal is initiated in the posterior polar follicle cells by activating a germ line protein kinase A (PKA) within the oocyte which then phosphorylates a target protein to further transmit the signal. The target protein remains uncertain but is speculated to be a MAP2 like protein which regulates the organization of microtubules within the oocyte (Sloboda et al., 1975; Theurkauf and Vallee, 1982; reviewed by Lane and Kalderon, 1994). The microtubules can then reorganize in mid-oogenesis and allow the anterior localization of *bcd* mRNA (reviewed by Lehmann,1995; Anderson, 1995). Based on the experimental data, the target protein could possibly be exu protein. Several pieces of evidence support this speculation. Firstly, exu protein is absolutely required for localization of *bcd* mRNA. Either lack of this protein or deletion of part of the protein would perturb localization of *bcd* mRNA. Secondly, exu protein is already localized in the oocyte at the early stages of oogenesis, reflecting a possibility of being phosphorylated by PKA within the oocyte. Another strong evidence is that exu is a phosphoprotein. The phosphorylation of exu protein is expected to be at multiple sites as at least four closely spaced major isoforms could be revealed by western blot analysis. Exu protein from the ovary extracts could be phosphorylated by several protein kinases including PKA (Cheung et al., unpublished data, manuscript in preparation). Hence the exu protein would possibly be a candidate of phosphorylation by PKA in the oocyte.

The phosphorylation of exu protein may be critical for its function to localize *bcd* mRNA. In the rescue mutant, Δ442-503, certain modification of exu protein was observed as two bands were revealed by western blot. The modification would probably be phosphorylation. No such observation was seen in the non-rescue mutants.

The component which received the localization signal from exu protein would probably be microtubules. Microtubules were found to be essential for localization of RNAs, *bcd* mRNA and *osk* mRNA in the oocyte (Pokrywka and Stephenson, 1991, 1994; Lane and Kalderon, 1994) and were reorganized just prior to localization of these RNAs (St. Johnston et al. 1989, 1991; Ephrussi et al. 1991; Kim-Ha et al. 1991). At early stages, a microtubule-organizing center (MTOC) was postulated to position at the posterior of the oocyte, sending microtubules toward the anterior and into nurse cells through cytoplasmic bridges (Theukauf et al., 1992, 1993; reviewed by Cooley and Theurkauf, 1994; St Johnston, 1995). The MTOC persists at the posterior until stage 6. By stage 7, the signals transmitted by PKA promotes degeneration of the posterior MTOC. At the same time, a new microtubule nucleating center appears at the anterior margin extending microtubules towards the posterior. Thus a uniform polarity of microtubules can be established for the localization of *bcd* mRNA.

The above model involves the initiation of a signal from the posterior polar follicle cells and the transmission of this signal through PKA to exu protein, and finally the destabilization of the posterior MTOC in the oocyte. However, this model is clearly incomplete. If the signal is transmitted through this single pathway, the observation of abnormal localization of *bcd* mRNA at both ends and *osk* mRNA at the middle of the oocyte in the PKA mutant will be expected to be seen in *exu* mutants. However, no transient localization of *bcd* mRNA at the posterior of the oocyte was observed in either exu^{sc} mutant or the *exu* deletion mutants. Thus the signaling pathway will not be a simple one but involve interactions with more components or pathways.

Nonetheless, according to the above model, exu protein will be expected to interact with microtubules in order to complete the signaling pathway. Hence, characterization of the associations between exu protein and microtubules would provide further insight into the mechanism of *bcd* mRNA localization.

Table 2.3 Deletion of the PEST domain in the deletion mutants

Deletion mutants	Rescue	Percentage of PEST deleted
Δ390-440	-	34 %
Δ442-503	+	56 %
Δ426-531	-	100 %
Δ390-531	-	100 %

Table 2.4 Comparison between the localization of exu protein and *bcd* mRNA in the deletion mutants

Deletion mutants	**Localization of exu protein**			**Localization of *bcd* mRNA**	
	stages 1-5	stages 6-8	stages 9-10A	stage 9	stage 10A
non-rescue mutants					
Δ302-375	-	+#	+	+*	-**
Δ374-388	-	+#	+	+*	-**
Δ390-440	-	+#	-	+*	-**
Δ426-531	-	+#	-	+*	-**
Δ54-193	-	-	-	-	-
Δ194-375	-	-	-	-	-
Δ390-531	-	-	-	-	-
rescue mutant					
Δ442-503	+	+	+	+	+

The level of localization in the oocytes was much lower than that of the rescue mutant.

* Only partial localization of *bcd* mRNA was observed. At stage 9, *bcd* mRNA was localized at anterior end of the oocyte but the localization appeared diffused.

** At stage 10A, *bcd* mRNA was distributed evenly but higher level of it was observed at anterior end of the oocyte and apical regions of the nurse cells.

Table 2.5 Percentage of deletion in the coding sequence of the deletion mutants

Deletion mutants	Rescue	Percentage of sequence deletion
Δ54-193	-	26 %
Δ194-375	-	34 %
Δ302-375	-	14 %
Δ374-388	-	3 %
Δ390-440	-	10 %
Δ442-503	+	12 %
Δ426-531	-	20 %
Δ390-531	-	27 %

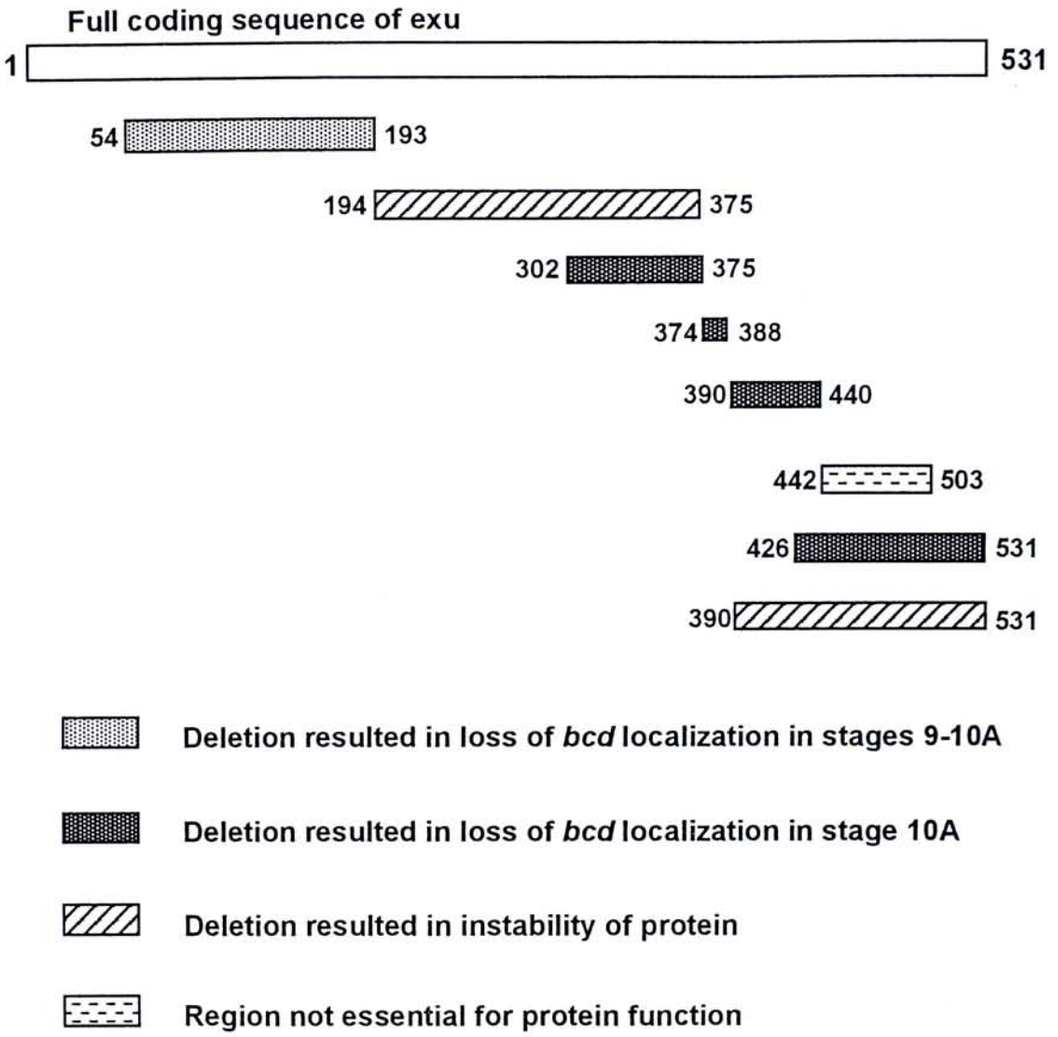


Figure 2.6 Potential functions of different regions of the *exu* coding sequence.

By deletion analysis, several regions of the coding sequences were found to confer different functions. Region of 54-193 amino acid residues seemed to important for localization of *bcd* mRNA in both stages 9 and 10A, while regions 302-442 and 503-531 amino acid residues were responsible for localization at stage 10A. Another two regions, which partially overlapped to the above regions, conferred stability of the protein.

CHAPTER 3

Determination of the interactions between exu and microtubules

3.1 Introduction

Microtubules have been demonstrated to play an essential role in localization of *bcd* mRNA. Pokrywka and Stephenson (1991) have shown that drugs, such as colchicine and taxol, which affect the stability of microtubules, disrupted the localization of *bcd* mRNA. They have further demonstrated that both *bcd* mRNA and exu protein are associated with a detergent insoluble fraction in the ovary extracts and the association can be released by microtubules destabilizing drugs (Pokrywka and Stephenson, 1994). Evidence of the dependence of localization of *bcd* mRNA on microtubules has been provided by the study of protein kinase A (PKA) mutations. Lane and Kalderon (1994) have characterized germ line PKA deficient mutants which disrupt both microtubules organization and RNA localization in the developing oocyte. In normal oocytes of stages 7 to 8, a microtubule organizing center (MTOC) is located at the anterior, establishing a uniform polarity of microtubule framework in which minus ends of microtubules are located at the anterior while plus ends are extending toward the posterior (Theurkarf et al., 1992). In this microtubule network, *bcd* mRNA is localized at the anterior pole while *osk* mRNA is localized at the posterior pole of the oocyte. However, in the PKA mutant, microtubule nucleating regions are located at both anterior and posterior poles, creating a network of microtubules with their minus ends situated at both anterior and posterior poles and their plus ends extended into middle of the oocyte. The abnormal localization of *bcd* mRNA at both poles and *osk* mRNA at the middle of the oocyte can be observed in the mutant. Thus, the distribution of RNAs is closely related to the polarity of microtubules whereby *bcd* mRNA is always localized at the minus ends while *osk* mRNA is always localized towards the plus ends of microtubules. This observation suggests that *bcd* mRNA is

transported by minus-end directed motors while *osk* mRNA is associated with plus-end directed motors (Ruohola et al., 1991; Clark et al., 1994; Lane and Kalderon, 1994).

As exu is associated with microtubules (Pokrywka and Stephenson, 1994) and is required for the localization of *bcd* mRNA (St. Johnston, et al., 1989), it is conceivable that exu protein may assist in localization of *bcd* mRNA through interactions with microtubules. Several approaches were used to characterize the interactions between exu protein and microtubules. The first analysis aimed at determining the *in vivo* dependence of distribution of exu protein on cytoskeleton based on pharmacological and cytological evidence (section 3.2). Further analysis intended to establish the biochemical evidence of the interactions. In section 3.3, the direct interactions between exu protein and microtubules were analyzed by immunoprecipitation. Sections 3.4 to 3.7 were experiments based on a cosedimentation approach, which depended on the affinity of microtubule-associated proteins (MAPs) to microtubules and the cosedimentation of these protein complexes. This cosediemntation approach was modified and refined and consequently, the method in section 3.7 has successfully determined that exu is, by definition, a MAP.

3.2 Localization of *bcd* mRNA and exu protein in the presence of drugs which destabilize cytoskeleton

To determine the *in vivo* dependence of localization of both *bcd* mRNA and exu protein on cytoskeleton, drugs which destabilize cytoskeleton were directly injected into the abdomen of wild type female flies and any alteration of the spatial and temporal distribution of *bcd* mRNA and exu protein were analyzed by *in situ* hybridization and immunohistochemical staining, respectively.

3.2.1 Materials and Methods

Stocks of colchicine was dissolved in absolute ethanol at a concentration of 20 mg/ml and cytochalasin D was dissolved in absolute ethanol at a concentration of 2 mg/ml. For injection, colchicine was diluted to 200 ug/ml in PBS and cytochalasin D was diluted to 20 ug/ml in PBS. Mock control was performed by injection of 1 % ethanol in PBS. A range of drug concentrations were used to ensure that the drug concentration was not limiting. A commercially available food dye solution was added to 10 % (v/v) to allow observation of the injection process. This ensured that the flies were properly injected as their abdomens took up the dye. The resulting drug solution was loaded into a microinjection needle and approximately 100 nl of drug solution was injected into the abdomen of each fly. The injected flies were allowed to recover for 17 to 20 hours at 25°C. Ovaries from flies were collected for immunohistochemical staining and *in situ* hybridization. The vials which contained the injected flies were incubated for fourteen days to determine the viability of their eggs.

3.2.2 Results

Adult flies were observed to emerge from the vials of the mock control and the cytochalasin D injection (drug concentration 20 ug/ml). These flies were healthy and normal without any observable morphological difference from the wild type. In contrast, no adult fly was emerged from eggs laid by the colchicine injected flies. Eggs were observed in the vials but none of them hatched into larvae.

The localization of exu protein in the mock treated egg chambers was indistinguishable from the wild type (Figure 3.1A). Exu protein was found to concentrate in oocytes of stage 2 up to stage 8 and to accumulate in apical regions of the nurse cells of stage 9 and 10A egg chambers. Therefore, the injection with 1% ethanol seemed to have no effect on the localization of exu protein.

The localization of exu protein was completely abolished in the colchicine treated egg chambers (Figure 3.1A). No localization of exu protein was observed at all stages of egg chambers (from germarium up to stage 9). In addition, neither stage 10A nor older egg chamber was found; only disintegrated egg chambers of comparable size to stage 10 egg chamber were seen. The early egg chambers seemed to also have a certain degree of disintegration in which the layer of follicle cells surrounding the oocyte were not complete (Fig. 3.1A, indicated by arrows). In these egg chambers, the effect of colchicine was not restricted the developing oocyte, but extended to the follicle cells. The stability of microtubules in the follicles cells may also have been affected, causing the discontinuous follicle cell layer at the early stages and even severe disintegration at the late stages (stage 10A).

The localization of exu protein was unaffected in the cytochalasin D treated egg chambers as comparing to the mock control. A range of drug concentration, 20, 40, and 80 ug/ml, was used to inject flies but there was no effect on the spatial and temporal distribution of exu protein. Thus, cytochalasin D seemed to have no effect on the localization of exu protein and hence actin filaments were not necessary for the localization.

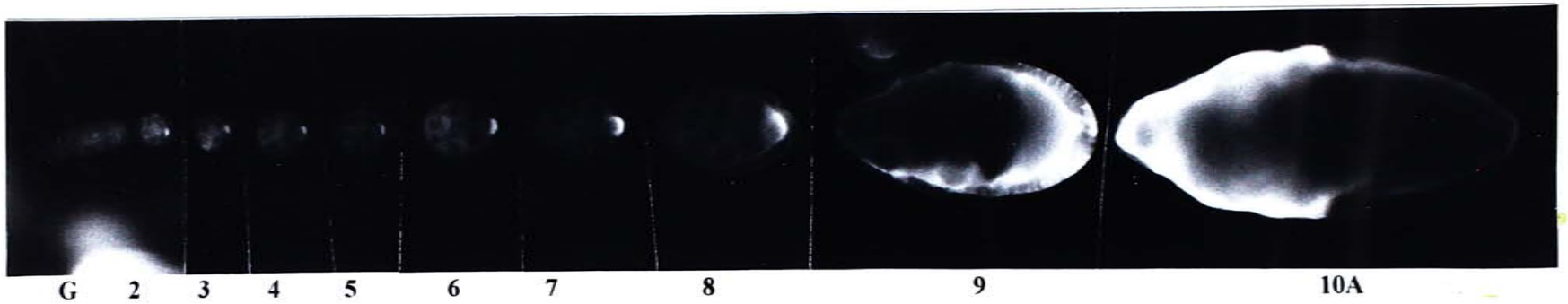
Using *in situ* hybridization techniques, the temporal and spatial distribution of *bcd* mRNA was analyzed in the drug-treated egg chambers. Localization of *bcd* mRNA was completely abolished in the colchicine-treated egg chambers (Figure 3.1B). The *bcd* mRNA was uniformly distributed in all stages of egg chambers. The

anterior localization in the oocyte was not seen from stages 6 to 9. As most of the stage 10A egg chambers tended to be disintegrated, the distribution of *bcd* mRNA was not detectable at stages older than stage 10A.

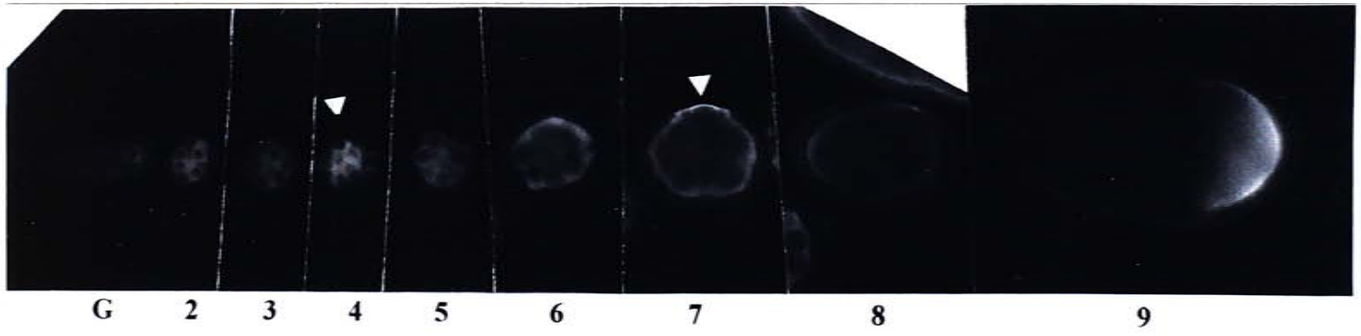
In the mock-treated and the cytochalasin D treated egg chambers, the distribution of *bcd* mRNA were similar to that of the wild type (Figure3.1B). Anterior localization at the oocyte was observed from stages 8 to 10A as well as the apical localization in the nurse cells in stage 10A. Therefore, cytochalasin D did not have any effect on the distribution of *bcd* mRNA.

Figure 3.1A Effects of microtubule and microfilament destabilizing drugs on the spatial and temporal expression of exu protein during oogenesis. The distribution of exu protein in the mock-treated egg chambers was indistinguishable from untreated wild type egg chambers. Localization of exu protein in colchicine-treated egg chambers was completely abolished. In addition to the effect on distribution of exu, the follicle cell layer covering the developing oocyte and the nurse cells was not complete and seemed to be disintegrated (indicating by arrows). On the contrary, the cytochalasin D-treated egg chambers were very similar to the mock-treated control and the destabilization of actin seemed to have no effect on the distribution of exu protein.

Mock-treated control



Colchicine treated



Cytochalasin D treated

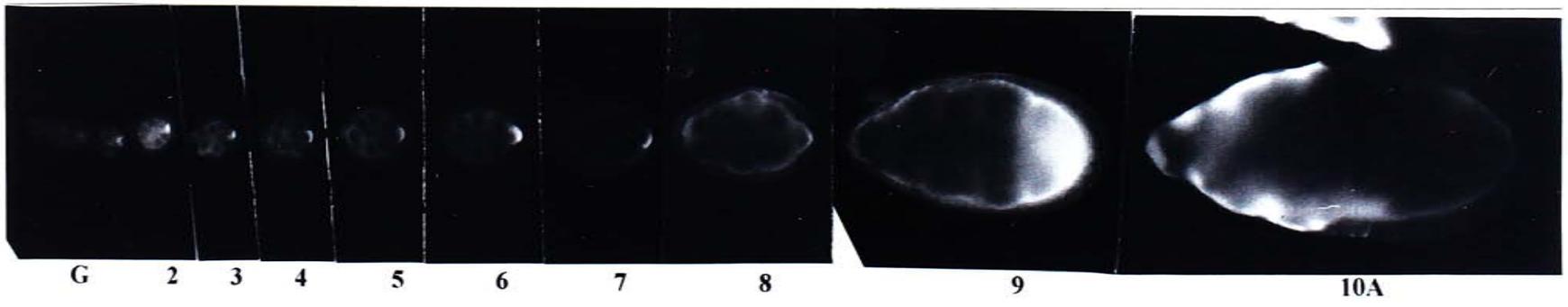
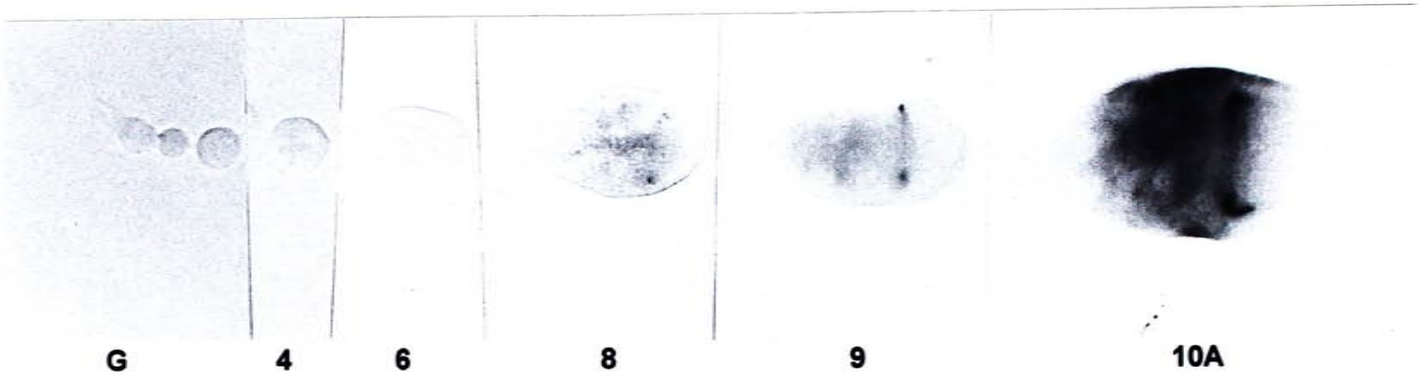
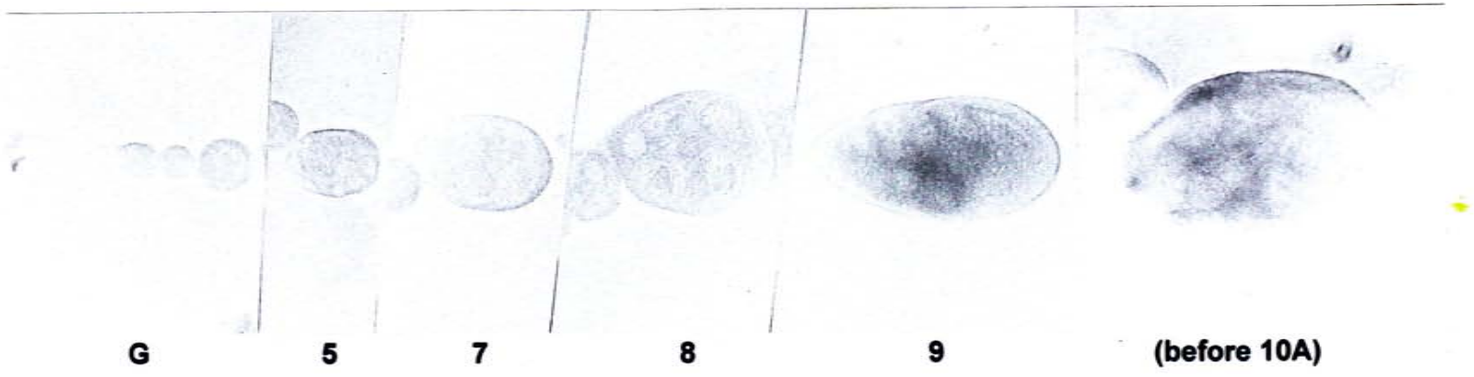


Figure 3.1B Effects of microtubule and microfilament drugs on spatial and temporal expression of *bcd* mRNA during oogenesis. Localization of *bcd* mRNA in colchicine-treated egg chambers was completely abolished; *bcd* mRNA was distributed evenly within the stages 8-10 egg chambers. Localization of *bcd* mRNA in cytochalasin D-treated egg chambers were very similar to that of the mock-treated control egg chambers.

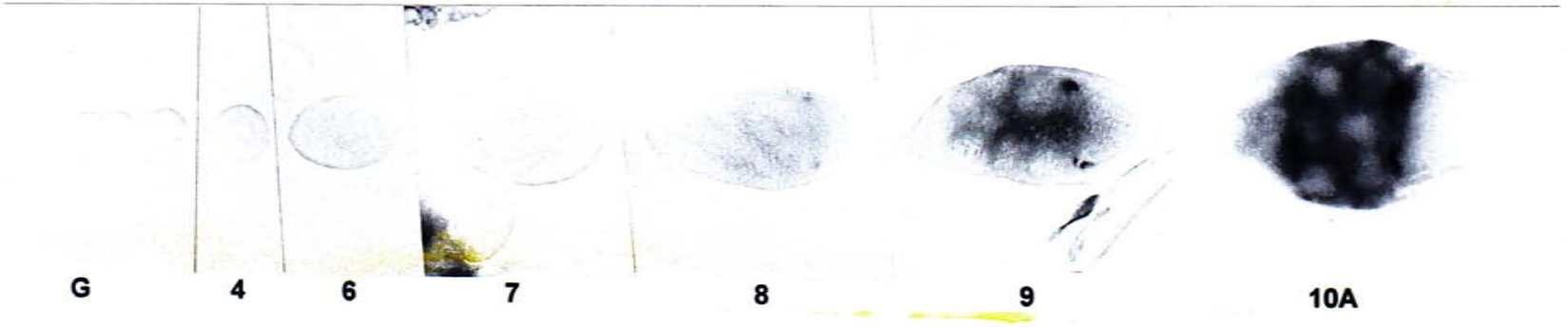
Mock-treated control



Colchicine treated



Cytochalasin D treated



3.3 Analysis of interactions between exu and microtubules by immunoprecipitation

The direct interaction between exu and microtubules was determined by incubating microtubules with exu protein which was isolated by immunoprecipitation in ovary homogenate. If direct binding occurred, microtubules would bind to the immunoprecipitation complex; otherwise, microtubules would remain unbound in the homogenate. The immunoprecipitation complexes were then analyzed for any associated microtubules by western blot analysis.

3.3.1 Materials and Methods

3.3.1.1 Immunoprecipitation of exu protein and binding of microtubules

Forty to fifty pairs of ovaries were isolated from two-day-old female flies in PBS. The ovaries were washed and homogenized with a disposable pestle in a microcentrifuge tube containing 0.5 ml of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 % NP40, 0.5 % DOC, 0.1 % SDS, 1.0 ug/ml pepstatin, 1.0 ug/ml leupeptin, 1.0 ug/ml aprotinin, 0.28 mM PMSF). The homogenate was transferred to a pre-chilled polypropylene tube. One milliliter of RIPA buffer was used to wash the microfuge tube and was pooled with the homogenate. The homogenate was sonicated on ice 3 times for 3 bursts at 10 s each. It was then divided into six 250 μl aliquots. For immunoprecipitation of exu protein, one aliquot of ovary homogenate was precipitated by incubating with 3 μl of anti-exu antibody for 2 hours and then with 50 μl of 10 % Pansorbin or Protein G-agarose (Calbiochem) for 90 minutes at 4°C with agitation. After the adsorption, the antigen-antibody (Ag-Ab) complex was collected by centrifugation at 4°C and was washed with 1 ml RIPA. The washing and centrifugation steps were repeated three more times. The final Ag-Ab complex was then washed and equilibrated in PM buffer (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT) three times for 5 minutes. The Ag-Ab complex in 0.5 ml PM buffer was supplemented with 20 uM taxol and 0.5 mM GTP and then with 0.2 mg/ml of taxol-stabilized microtubules. After incubation for 30 minutes at room temperature, the Ag-Ab complex was

recovered by centrifugation and washed three times in PM buffer for 10 minutes while the supernatant was acetone-precipitated by adding of 2 volumes of ice-cold acetone and incubating for 1 hour on ice. Both of the Ag-Ab complex and the pellet of acetone-precipitated supernatant were resuspended in 25 μl of SDS sample buffer and analyzed by Western blot analysis. Two duplicated blots were produced. One blot was detected with a rabbit anti-exu antibody and then an AP-conjugated goat anti-rabbit antibody. The other was detected with a mouse monoclonal anti-β-tubulin antibody (Boehringer) and then an AP-conjugated anti-mouse antibody (Tropix) as the secondary antibody.

3.3.1.2 Purification of tubulin from bovine or rat brains

Tubulin was purified by a twice-cycled polymerization-depolymerization protocol described by Williams (1992). Bovine or rat brains were minced into small pieces in PM buffer on ice and then washed two times in the same buffer. Using a 30 ml-glass homogenizer with a telfon pestle, the brains were homogenized in 30 ml of PM per 100 g of tissue on ice. All subsequent steps were performed at 4°C. The homogenate was clarified by centrifugation at 6,500 x g for 15 minutes in a JA-20 rotor in a Beckman J-2 MC centrifuge. The supernatant was decanted and centrifuged again at 96,000 x g for 75 minutes in a TLA-120.2 rotor in Beckman Optima TLX Tabletop Ultracentrifuge or a Type 42.1 rotor in a Beckman L7-55 Centrifuge. The supernatant was saved and an equal volume of PM-8M (PM buffer, 8 M glycerol) was added to make a final concentration of 4M of glycerol. In order to promote microtubules formation, the supernatant was supplemented with a final concentration of 1 mM GTP and 2.5 mM ATP and incubated for 45 minutes at 34°C. (A high concentration of ATP was required to inhibit the binding of motor proteins to microtubules). After centrifugation at 96,000 x g for 60 minutes at 30°C, the supernatant was discarded and the pellet, which contained microtubules, was resuspended in 1/4 of the original volume of PM. The pellets were subjected to five gentle strokes in a Dounce homogenizer to produce a uniform suspension. The suspension was incubated for 30 minutes on ice to allow microtubules depolymerization. Centrifugation at 96,000 g for 60 minutes at 4°C resulted in tubulin subunits in the supernatant and un-depolymerized microtubules as well as

impurities in the pellet. The supernatant was saved and added with an equal volume of PM-8M to make a final concentration of 4M of glycerol. Then a second cycle of polymerization, centrifugation, depolymerization and centrifugation was carried out by repeating the above steps. After the depolymerization and centrifugation, the supernatant, referred to as twice-cycled microtubule protein, was saved and stored in aliquots at -70°C as stocks of tubulin.

3.3.1.3 Determination of protein concentration of the tubulin stock by Folin-Lowry method

The protein determination method was adapted from Lowry et al. (1951). A volume of 0.1 ml protein sample was added with an equal volume of 2 N NaOH. Another set was made by BSA of known concentrations as standards. After a 30 minute incubation period at room temperature, serial dilutions of the denatured protein samples were made by diluting with 1 N NaOH. Each dilution was mixed with 1 ml of Reagent A (prepared fleshly by mixing 100 ml 2 % Na_2CO_3, 2 ml 1 % $CuSO_4$ and 2 ml 2 % K-Na tartrate) and incubated for 10 minutes. Each dilution was added with 100 µl of 1 N of folin and vortexed immediately. The absorbance at OD_{750} was determined spectrophotometrically between the period of 20 and 45 minutes after the addition of folin. A BSA standard curve was established and the concentration of the protein was read from the plot.

3.3.1.4 Taxol-stabilized microtubules

Microtubules were polymerized from tubulin subunits by the method described by Arshad Desai (personal communication) . Tubulin stock was diluted to 2 mg/ml in PM buffer and supplemented with 1 mM GTP on ice. The formation of microtubules from tubulin was promoted by high temperature and a serial addition of taxol. The diluted tubulin was warmed to 37 °C and supplemented with 1/100 volume of 0.02 mM taxol in DMSO. After incubation for 5 minutes, an additional 1/ 100 volume of 0.2 mM taxol in DMSO was added and incubated for 5 minutes. The final concentration of taxol was made to about 20 uM by further addition of 1/100 volume of 2 mM taxol in DMSO and the microtubules was incubated for 15 minutes.

3.3.2 Results

Among the four sets of immunoprecipitation complexes, exu protein was only found in the wild type ovary extracts (lane 1, Figure 3.2A), although the amount was low. If microtubules have bound to exu protein, tubulin would be expected to be present only in the immunoprecipitation complex of the wild type ovary extracts. However, microtubules were found to bind the immunoprecipitation complex nonspecifically as tubulin bands were present in all the lanes including those controls without exu protein (Figure 3.2B). Tubulin was found to be present in the Ag-Ab complex independent of the types of extracts (the wild type, lane 1; exu^{sc} ovary extract, lane 2, Figure 3.2A,B). In addition, the microtubules was most likely bound to the protein G-agarose as tubulin was also present at the protein G-agarose only complex (lane 4, Figure 3.2A,B). Therefore, this immunoprepicitation and binding method was unable to determine the interactions between exu protein and microtubules.

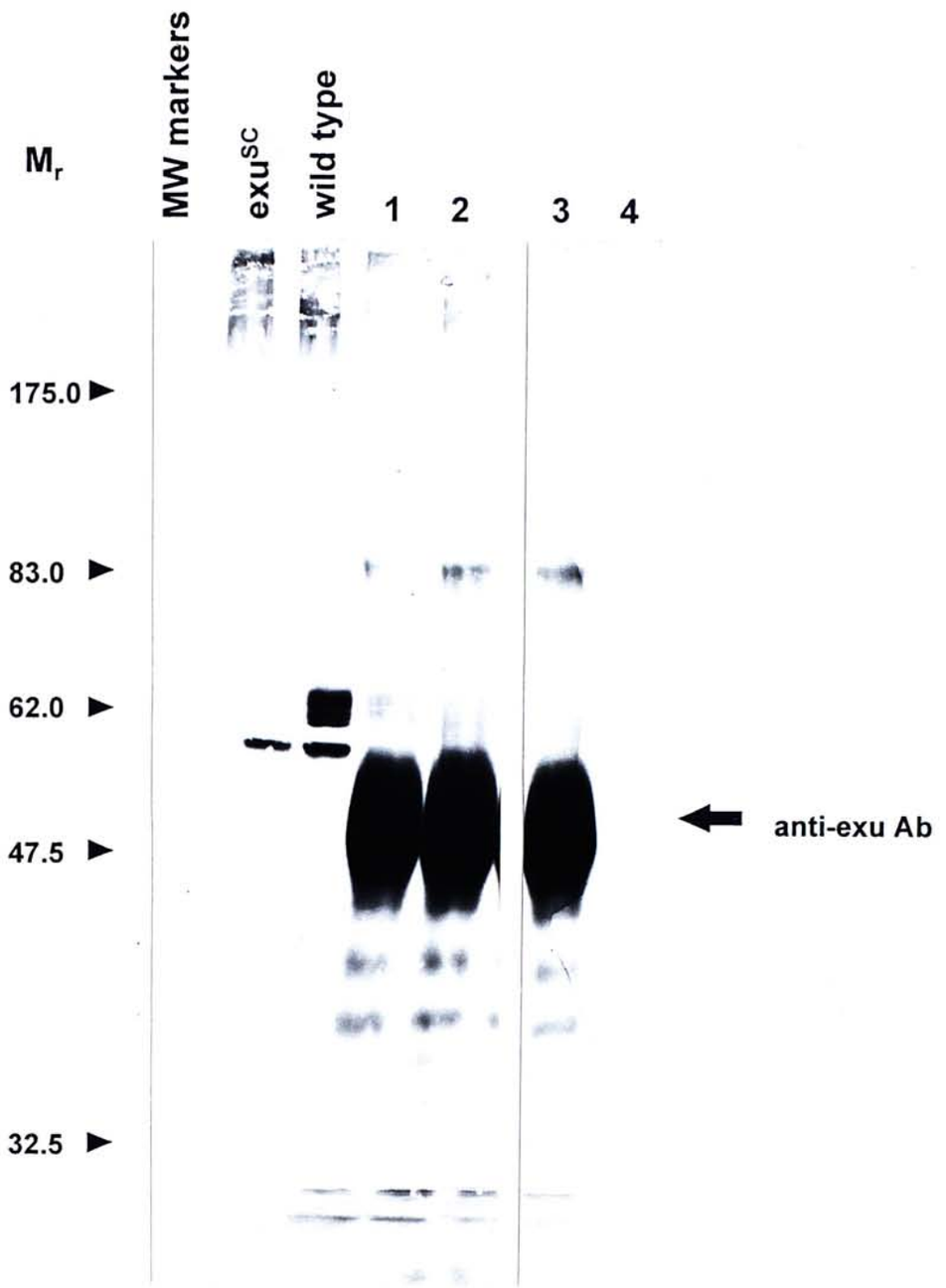


Figure 3.2 Detection of exu protein and microtubules in the immnuoprecipitation complexes.

A. Western blot analysis of exu protein in immunoprecipitation complexes. Multiple isoforms of exu protein can be detected in the wild type ovary extracts. As a negative control, the ovary extracts of the *exu*^{sc} protein null mutant was run in parallel with that of the wild type. The arrow indicates the position of the rabbit anti-exu antibody that was detected by the AP-goat anti-rabbit antibody.

Lane: **exu**^{sc}, ovary extracts of the protein null mutant; **wild type**, ovary extracts of the wild type; **1**, immunoprecipitation complex made by incubating wild type extracts, anti-exu antibody(Ab), protein G-agarose(G); **2**, immunoprecipitation complex made by incubating *exu*^{sc} extracts, Ab, G; **3**, immunoprecipitation complex made by incubating Ab and G only, no extracts; **4**, immunoprecipitation complex made by incubating G only, with neither extracts nor Ab. All immunoprecipitation complexes were supplemented with microtubules.

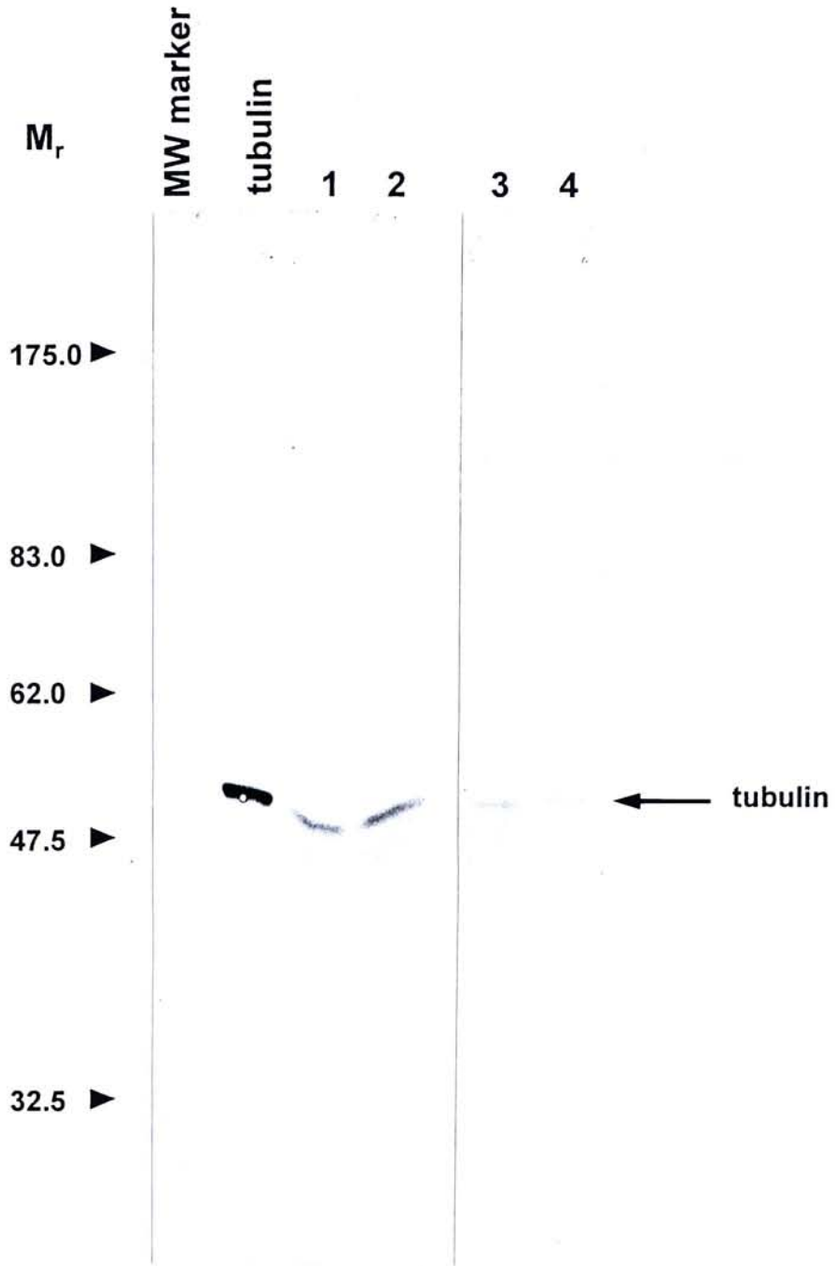


Figure 3.2 Detection of exu proteinin and microtubules in the immnuocomplexes.

B. Western blot analysis of tubulin in Immunoprecipitation complexes. A duplicated blot of Figure 3.2A was detected by anti-β-tubulin antibody. Microtubules were denatured into tubulin monomers on SDS PAGE and tubulin was detected as a molecular weight of 55 (indicated by arrow).

Lane: **tubulin**, purified tubulin was run alongside as a marker; **1-4**, see Figure 3.2 A.

3.4 Analysis of interactions between exu and microtubules by cosedimentation

The sedimentation characteristics of microtubules (Weisenberg, 1972; Shelanski, et al., 1973), can be used to determine potential interactions between exu protein and microtubules. This was achieved by incubation of ovary extracts with microtubules and by centrifugation to co-sediment microtubules and microtubule associated proteins (MAPs) (Figure 3.3). MAPs were first described as proteins that co-purified with tubulin during repetitive cycles of assembly and disassembly (Borisy et al., 1975; Wiche, 1989). In addition, some associated proteins display transient interactions with microtubules, such as motor proteins are usually classified as microtubule binding proteins (MTBPs) (Richard and Kreis, 1991). The method was originally used for the isolation of MAPs and hence exu could be classified as a MAP if the cosedimentation of exu protein and microtubules occurred.

3.4.1 Materials and Methods

The method was adapted from several microtubule and MAPs cosedimentation protocols (Yang et al., 1989; Aizawa et al., 1991; Rickard and Kreis, 1991; Michalik et al., 1993; Hays, et al., 1994; Li, et al., 1994; Walczak, et al., 1996), with the following modifications. About 80 pairs of ovaries were isolated from female flies and washed in PM buffer. The ovaries were homogenized with a dounce homogenizer in 1 ml of PM buffer. The ovary homogenate was first clarified by centrifugation at 37,000 r.p.m. for 30 minutes in a Beckman TLA-120.2 rotor. The supernatant was decanted and supplemented with 0.5 mM GTP and protease inhibitors (1 ug/ml pepstatin A, 1 ug/ml leupeptin, 1 ug/ml aprotinin) before warming up to room temperature. The extract was added with 5 uM of taxol in DMSO and incubated for 5 minutes at 34°C. An additional 15 uM taxol was added and the extract was further incubated for 10 minutes. Taxol-stabilized microtubules were supplemented to a final concentration of 0.2 mg/ml and the binding was allowed at 34°C for 30 minutes. By loading the extracts onto 1 ml of 30 % glycerol

cushion in PM buffer with 10 uM taxol and protease inhibitors, the microtubules was collected by centrifugation at 33,000 r.p.m. for 45 minutes in a Beckman TLS-55 swinging bucket rotor. The pellets were collected while the supernatants were concentrated by acetone precipitation. Both the pellets and supernatants were analyzed by SDS PAGE and western blot analysis as described in sections 2.3.1.2 and 2.3.1.3.

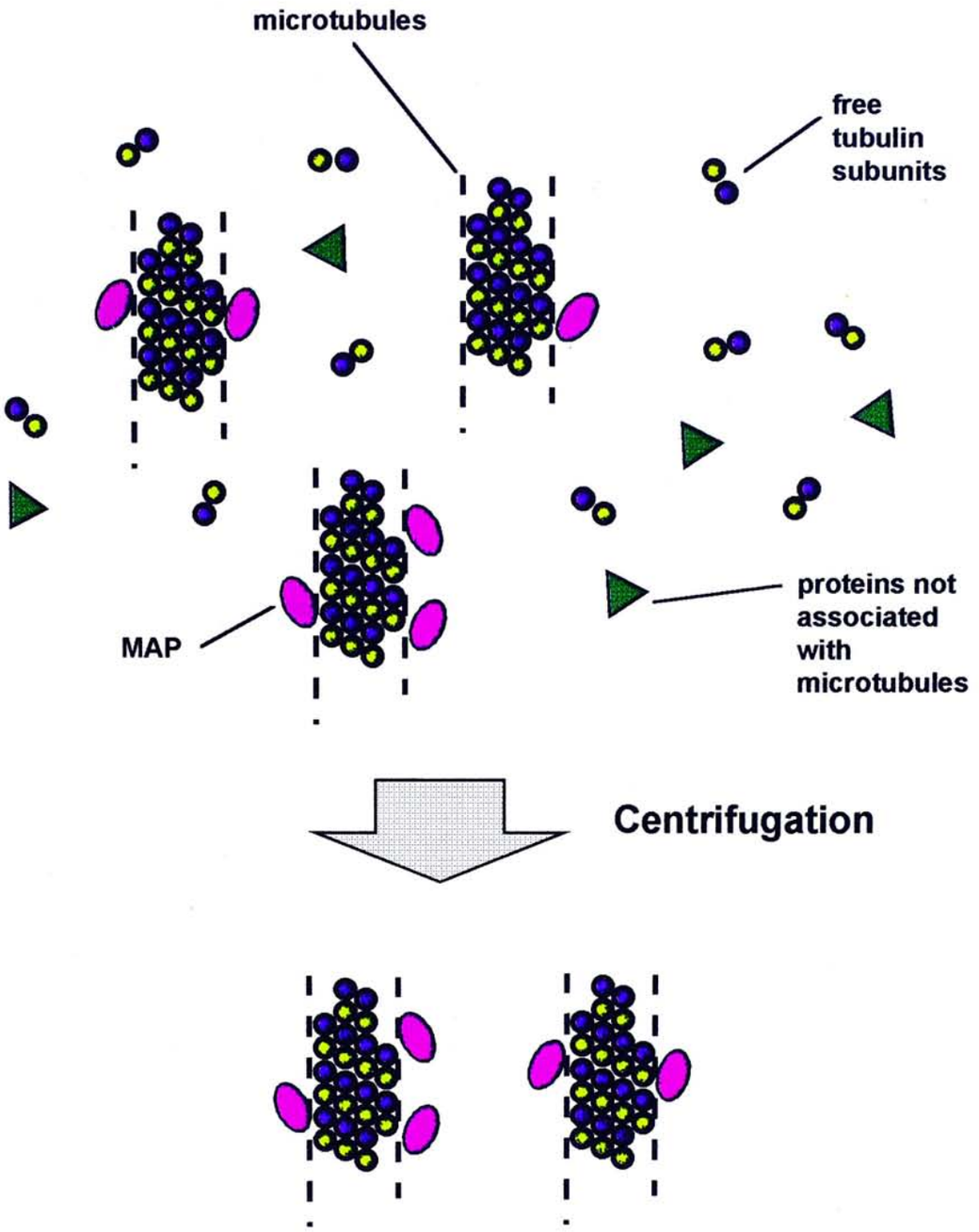


Figure 3.3 Basic principle of characterization and purification of MAPs by cosedimentation

Ovary extracts are incubated with microtubules and MAPs bind on microtubules while other proteins including free tubulin subunits are remained unbound. By centrifugation, microtubules and MAPs can be isolated in the pellet leaving other proteins in the supernatant.

3.4.2 Results

If exu protein were associated with microtubules, it would cosediment with microtubules and could be found in the pellet. In the wild type ovary extracts supplemented with exogenous microtubules (lane P1, Figure 3.4A), exu protein was found in the microtubule pellet. However, the same observation was found in the wild type ovary extract without the addition of microtubules (lane P3), although the amount of exu protein at lane P3 was much lower than that in lane P1. Therefore, the amount of exu protein sedimented was higher in the presence of exogenous microtubules. This implied that exu protein was cosedimented with microtubules, suggesting their interactions. The presence of a small amount of exu protein in lane P3 would be due to the incomplete clarification of the ovary extract during initial steps. Alternatively, association of exu protein to the endogenous microtubules might be sufficient for sedimenting exu protein.

In addition, it was noted that most of exu protein was lost in the clarification step. The exu protein in the pellet of clarification of ovary extracts (lanes 5) was much more abundant than that in lanes P1 and S1. Most of the exu protein was not extracted into soluble form but retained in the insoluble fraction of the ovary extracts. Thus the homogenization method described here was not sufficient to release exu protein.

As shown in Figure 3.4B, tubulin was found in both the pellets and the supernatants. As the assembly of microtubules continued to a steady state at which the microtubules mass and the concentration of non-polymerized tubulin subunits remained constant (Bayley et al., 1994), microtubules were sedimented while the free tubulin subunits remained in the supernatant after centrifugation. The abundance of tubulin indicated that the pellet was the result of sedimentation of microtubules which were denatured into tubulin monomers on SDS PAGE.

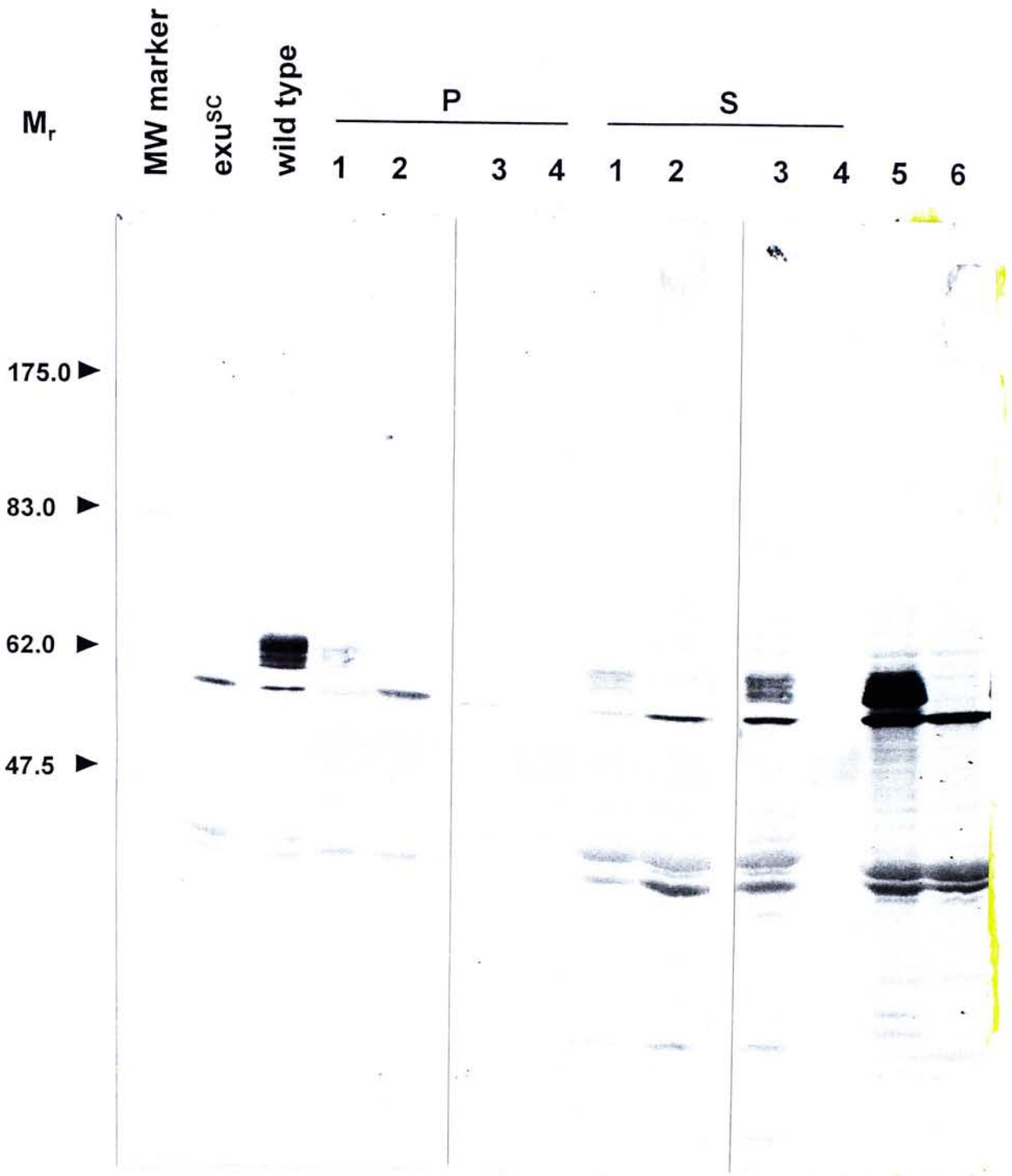


Figure 3.4 Analysis of exu protein cosedimented with microtubules.

After incubation of with ovary extract, microtubules were pelleted by centrifugation and analysed for cosedimented exu protein.

A. Western blot analysis of exu protien cosedimented with microtubules

Lane: **exu^{sc}**, **wild type**, ovary extracts from *exu^{sc}* and the wild type respectively; **P**, **S**, pellets and supernatants resulting from centrifugation after the incubation of **1**, wild type ovary extracts and microtubules (MT); **2**, *exu^{sc}* extracts and MT; **3**, wild type extracts only, no MT; **4**, MT only, no extracts. **5**, **6**, clarification pellets of the extracts of the wild type and *exu^{sc}* respectively.

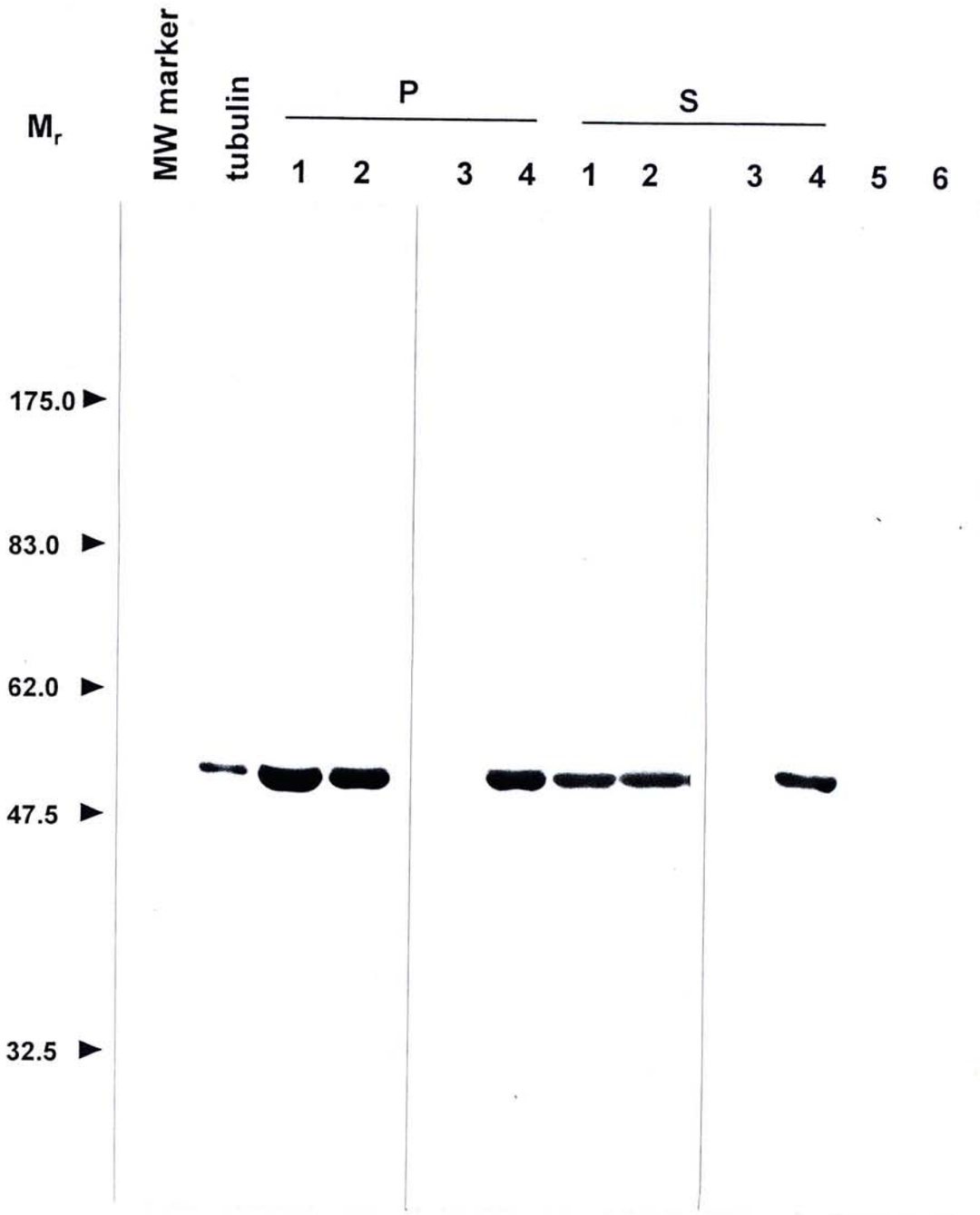


Figure 3.4 Analysis of exu protein cosedimented with microtubules.

B. Western blot analysis of tubulin. A duplicated blot of Figure 3.4A was detected by anti-β-tubulin antibody.

Lane: **tubulin**, purified tubulin was run alongside as a marker; **P1-P4**, **S1-S4**, **5**, **6**, see Figure 3.4 A.

3.5 Analysis of interactions between exu and microtubules using detergent extracted ovary extract for cosedimentation

As there were difficulties in extracting exu protein described in the previous section (section 3.3), detergents was supplemented to facilitate better solubilization of proteins from ovary extracts. The detergent-containing RIPA buffer used in the immunoprecipitation protocol was adopted to homogenize ovaries as it was useful to release exu protein from the ovary lysate. Followed by dialysis, the homogenate was equilibrated with a microtubule-favorable buffer and was supplemented with exogenous microtubules for the cosedimentation experiment.

3.5.1 Materials and Methods

Ovaries were homogenized in RIPA buffer on ice. The homogenate was sonicated three times for 10 seconds and then clarified by centrifugation at 57,000 r.p.m. for 45 minutes at 4°C in a Beckman TLA-120.2 rotor. A surface lipid layer was removed and the supernatant was dialyzed in 1 liter of RIPA buffer without detergents (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA), plus 0.28 mM PMSF, 5 mM NaF, 100 uM Na_3VO_4, for 4 hours at 4°C. Dialysis was repeated once more overnight with a change of fresh buffer and then a final change of buffer for 4 hours with BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA) (Walczak et al., 1996; Arshad Desai, personal communication). The extracts was kept at -70°C. The cosedimentation procedure has been described in section 3.4.1.

3.5.2 Results

As shown in Figure 3.5A, exu protein was found in both pellets of the wild type extracts with (lane P1) and without (lane P3) the addition of exogenous microtubules. Therefore, exu protein seemed to be sedimented into the pellets independent of the presence of microtubules. However, a higher amount of exu protein was sedimented in the presence (lane P1) than in the absence (lane P3) of exogenous microtubules suggesting that exu might have interactions with microtubules.

Besides, most of the exu protein from the ovary extracts was lost during the clarification (lane P5). Thus, exu protein was not released into soluble forms and again the detergent extraction method was not sufficient to extract exu protein.

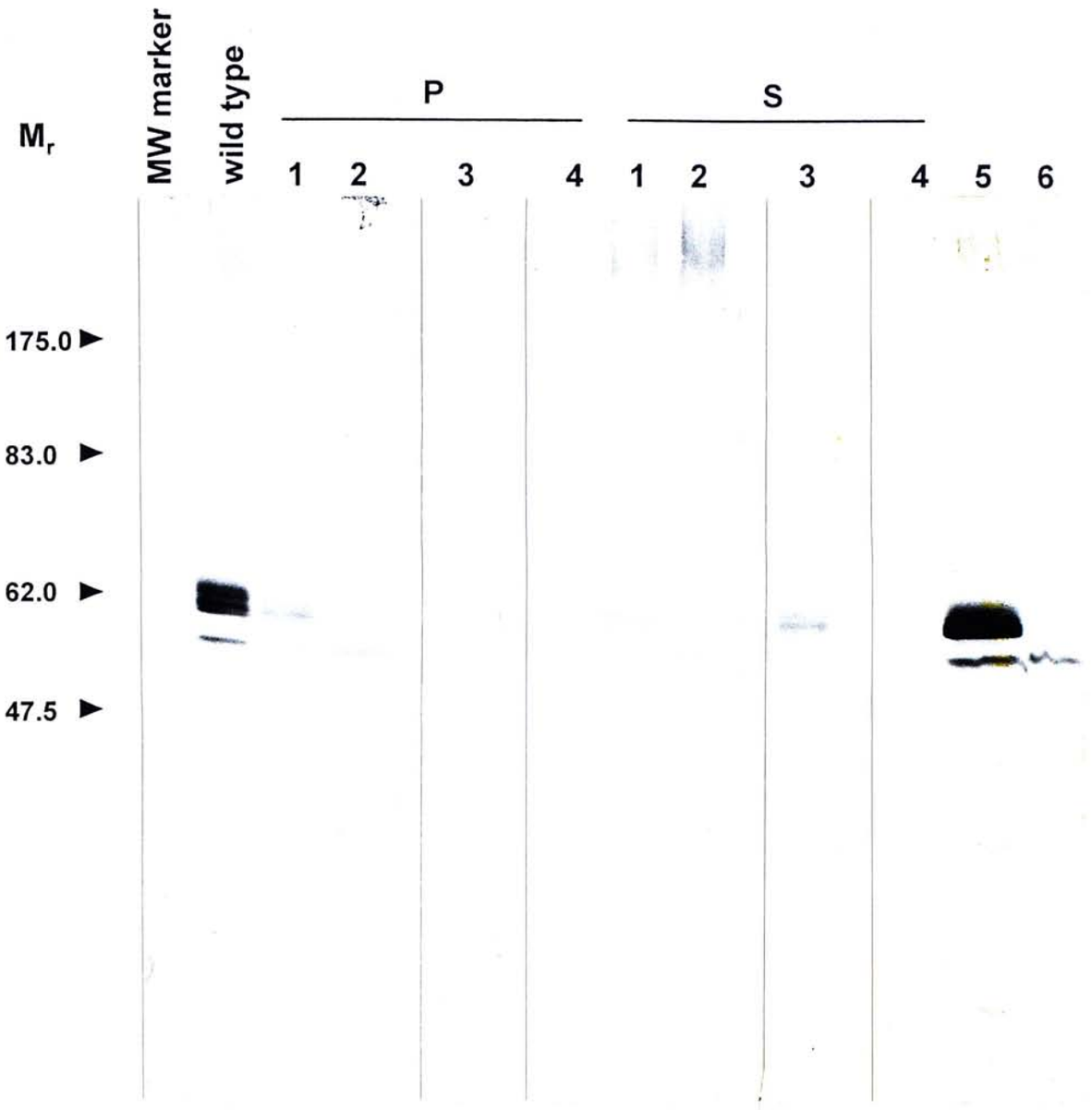


Figure 3.5 Cosedimentation of exu protein and microtubules using detergent extracted ovary lysate.

Ovaries were extracted in detergent-containing buffer and then dialysed to exclude detergents before the cosedimentation experiment.

A. Western blot analysis of exu protein.

Lane: **wild type**, ovary extracts of the wild type; **P**, **S**, pellets and supernatants resulting from centrifugation after the incubation of **1**, wild type ovary extracts and taxol-stabilized microtubules (MT); **2**, exu^{sc} extracts and MT; **3**, wild type extracts only, no MT; **4**, MT only, no extracts. **5**, **6**, clarification pellets of the extracts of the wild type and exu^{sc} respectively.

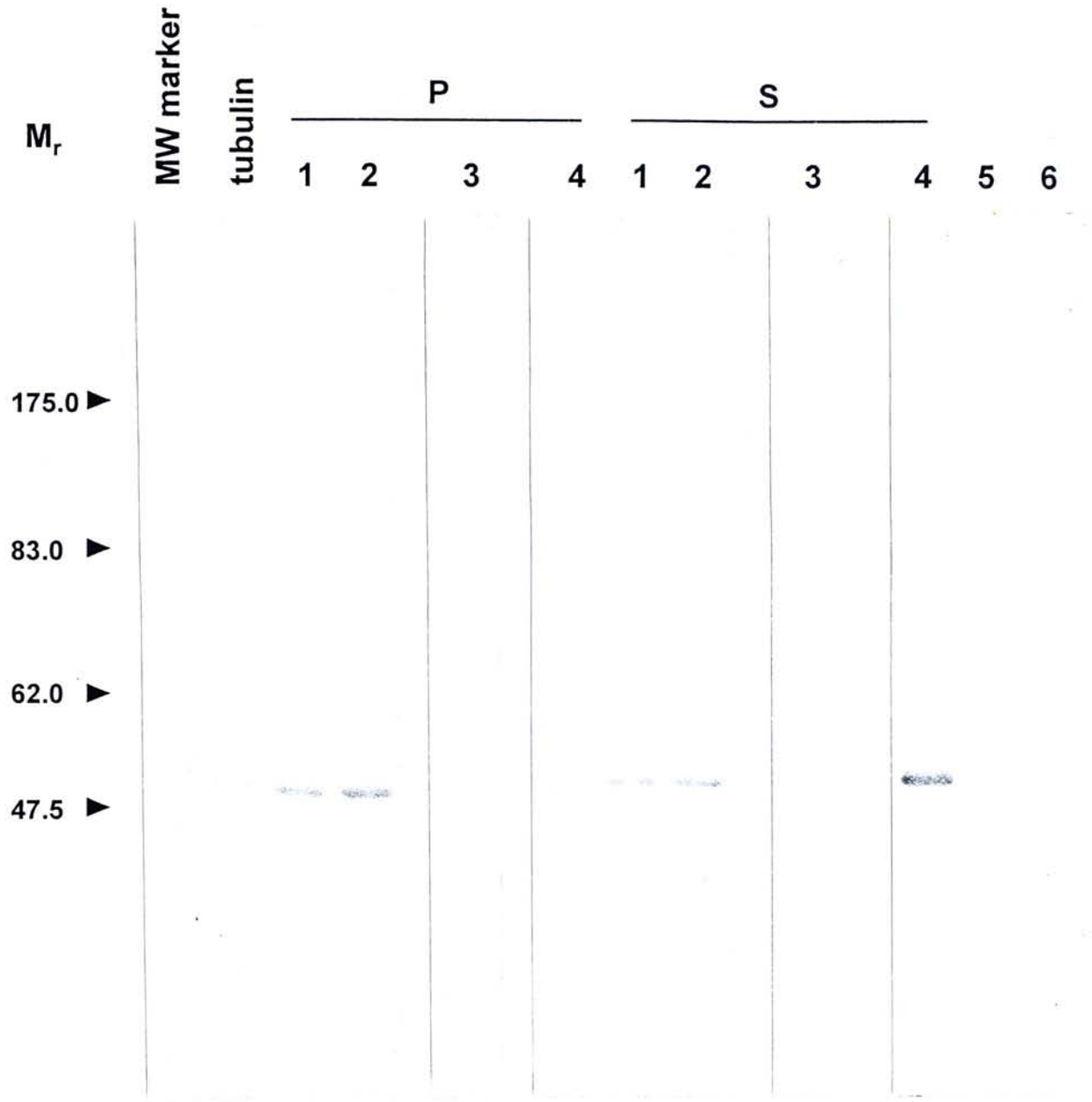


Figure 3.5 Cosedimentation of exu protein and microtubules using detergent extracted ovary lysate.

B. Western blot analysis tubulin. A duplicated blot of Figure 3.5A was detected by anti-β-tubulin antibody.

Lane: **tubulin**, tubulin was run alongside as a marker; **P1-P4**, **S1-S4**, **5**, **6**, see Figure 3.5 A.

3.6 Analysis of intracellular distribution of exu protein and Release of exu protein by sodium carbonate treatment for cosedimentation with microtubules

As only low level of exu protein was extracted even in the presence of detergents, subcellular fractionation was used to analyze the subcellular distribution of exu protein. A sodium carbonate treatment was adopted to release exu protein from the microsomal fraction for the cosedimentation experiment. This treatment was found to be effectively removing ribosomes from endoplasmic reticulum membranes and converting sealed vesicles into flat membrane sheets, resulting in release of cisternal contents (Fujiki et al., 1982). Besides, this method was non-destructive and had a higher efficiency in releasing proteins than normal low detergent conditions. Exu protein released by this treatment was used for cosedimentation experiment and the associations between exu and microtubules were verified by western blot analysis.

3.6.1 Materials and Methods

3.6.1.1 Subcellular fractionation of ovary extracts

Ovaries were homogenized and fractionated according to the protocol for fractionation of liver (Evans, 1992) (Figure 3.6). The ovaries (about 100 pairs) were isolated from well-fed two-day-old flies in PBS. They were briefly washed in PM and resuspended in 1 ml of the same buffer. Using a tight-fit pestle, the ovaries were dounce-homogenized by 20 to 30 strokes on ice. All subsequent steps were performed at 4°C. The homogenate was first clarified by centrifugation at 1000 x g for 10 minutes in a JA-20 rotor in Beckman J-2 MC centrifuge. The supernatant was saved while the pellet was resuspended in 0.5 ml PM. The suspension was centrifuged once again and the pellet was collected as F1 fraction which contained mostly of large membranes and nuclei. The supernatant was pooled with the previous one and was centrifuged under the same conditions as above. This latter pellet, which contained very low amount of cellular components, was discarded. The supernatant was spun at 8,000 x g for 20 minutes and the resulting supernatant

was saved while the pellet was resuspended in 0.5 ml PM and was collected by centrifugation. The supernatant was pooled while the pellet was again resuspended and collected by centrifugation. The resulting pellet was referred as an F2 fraction which contained organelles such as lysosomes, mitochondria and peroxisomes. The supernatant was pooled and centrifuged at 100,000 x g for 1 hour in a TLA-120.2 fixed angle rotor in Beckman Optima TLX Tabletop Ultracentrifuge. This final pellet was a microsomal fraction and was referred to as the F3 fraction.

3.6.1.2 Release of contents from fractions by sodium carbonate treatment

Three fractions, F1, F2 and F3 were collected as described above. Each fraction was divided into two portions: one was resuspended in 1 ml of 100 mM sodium carbonate (pH 11.5) while the other was resuspended in 1 ml of PM as a control. The sodium carbonate treatment converts closed vesicles to open membranes in the cellular fraction and releases their content proteins and peripheral membrane proteins (Fujiki et al., 1982). The suspensions were incubated for 30 minutes at 4°C. After centrifugation at 230,000 x g for 1 hour at 4°C in the TLA-120.2 rotor, both pellets and supernatants were saved for analysis.

3.6.1.3 Cosedimentation of exu proteins with microtubules

The supernatant from section 3.6.1.2 was dialyzed in 1 liter of PM buffer with 0.28 mM PMSF, 5 mM NaF, 100 uM Na_3VO_4 for 4 hours and repeated once with fresh buffer for 4 hours at 4°C. After dialysis, the extract was clarified by centrifugation at 78,000 r.p.m. in the Beckman TLA-120.2 rotor.

The interactions between exu protein and microtubules was determined by the microtubules sedimentation protocol described by Arshad Desai (personal communication) and Walczak et al. (1996) with the following modifications. The extract was supplemented with protease inhibitors and 1 mM GTP and warmed to room temperature. The extracts was further supplemented with 20 uM taxol and then 0.2 mg/ml of taxol-stabilized microtubules and incubated for 20 minutes at 30°C. The microtubules and microtubule associated proteins were collected by centrifugation on 1ml cushion of 40 % glycerol in PM buffer, containing 10 uM taxol, protease inhibitors and 5 mM NaF, 100 uM Na_3VO_4, at 42,000 r.p.m. for 30

minutes at 30°C in a Beckman TLS-55 swinging bucket rotor. Both the pellets and supernatants were analyzed by SDS PAGE and western blot as described in sections 2.3.1.2 and 2.3.1.3.

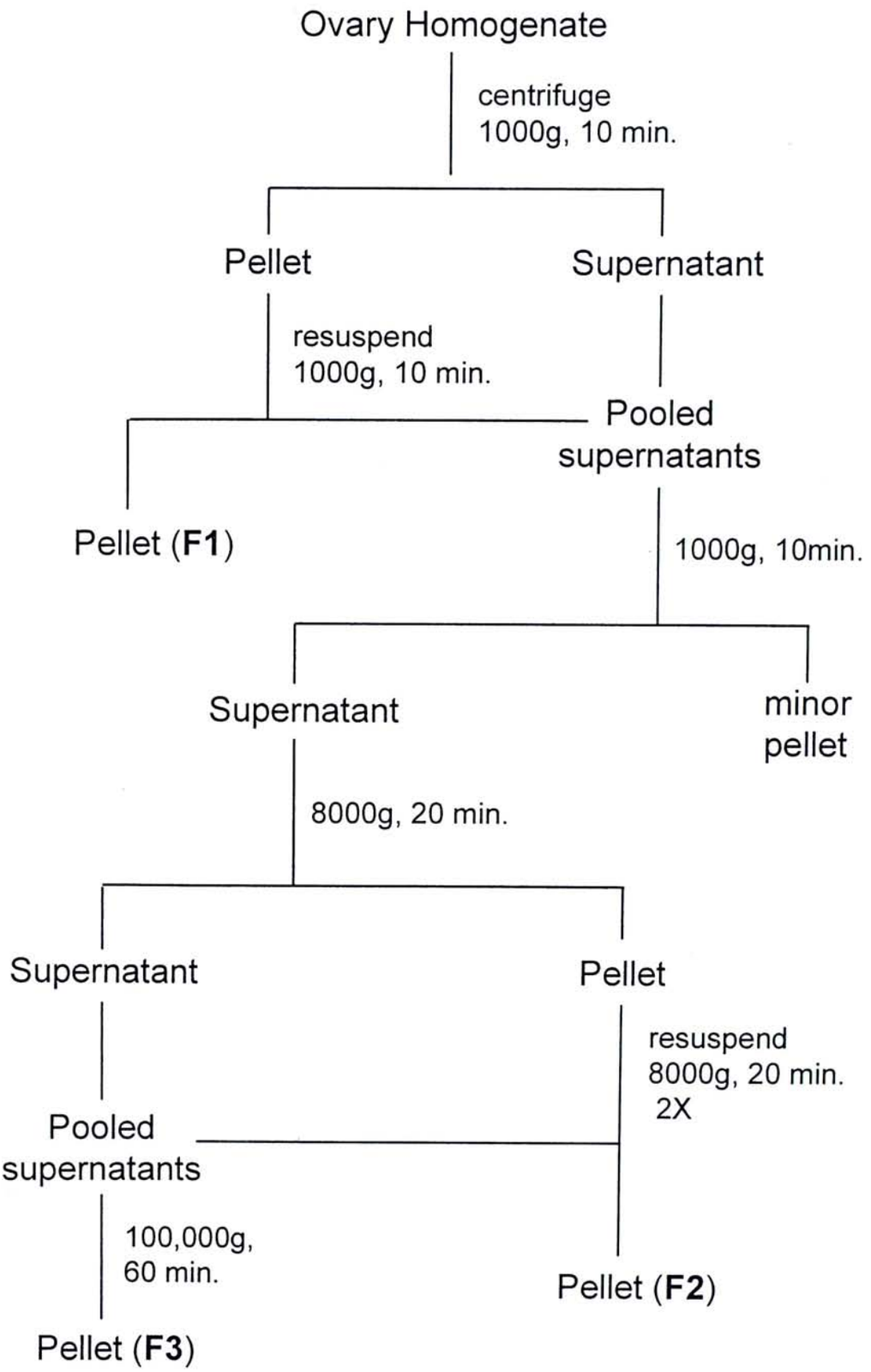


Figure 3.6 A diagrammatic representation of the subcellular fractionation of ovary

3.6.2 Results

3.6.2.1 Intracellular distribution of exu protein

Brief homogenization process was unable to extract the exu protein from the ovary homogenate. Hence most of exu protein was lost during the clarification step in the methods in the previous sections. A more effective method was employed to release exu protein. As shown in Figure 3.7, most of exu protein was found in fraction F3, some was present in fraction F1, and little in fraction F2. The fraction F3, which was referred to as the microsomal fraction, contained mostly of cellular microsomes (Evans, 1992).

Furthermore, the exu protein in fraction F3 was released into a soluble form after the Na_2CO_3 extraction. In the controls which were extracted with PM buffer, most of exu protein remained insoluble in the pellet (lane P-control) and little was released in the supernatant (lane S-control). On the contrary, much of the exu protein was released in the supernatant (lane S-Na_2CO_3) and only some remained in the pellet (lane P-Na_2CO_3) after the Na_2CO_3 treatment. The treatment of Na_2CO_3 appeared to be able to release significant amount of exu protein.

3.6.2.2 Cosedimentation of exu protein with microtubules using Na_2CO_3 released extracts

As shown in Figure 3.8A, most of the exu protein was found in the supernatants (lanes S1-S4), where exu protein did not associate with microtubules. Although a comparatively larger amount of exu protein was extracted, there was only a very low level of exu protein cosedimented with microtubules. However, we still could not conclude that exu protein did not bind microtubules because the processes of sodium carbonate treatment and dialysis would possibly alter the biochemical properties of the proteins. The resulting exu protein would be modified and might have lost the ability to associate with microtubules.

To determine if microtubules were sedimented, microtubules were supplemented and so abundant tubulin was observed in the pellets (lanes P1 and P3, Figure 3.8B) while a low level was found in the supernatants (lanes S1 and S3). (Microtubules are polymerized form of tubulin subunits and they are depolymerized on SDS PAGE.) The presence of the tubulin band in supernatants was due to the

existence of the steady state at which the microtubules mass and the concentration of non-polymerized tubulin subunits remained constant (Bayley et al., 1994). As an equilibrium existed between tubulin subunits and microtubules, microtubules were not exclusively present in the pellets (lanes P1 and P3). On the contrary, a very low level of tubulin was found in the pellet (lane P4) and most remained in the supernatant (lane S4) as only unpolymerized tubulin subunits was added. In lanes P2 and S2, no detectable tubulin was detected as neither mictoubule nor tubulin was supplemented.

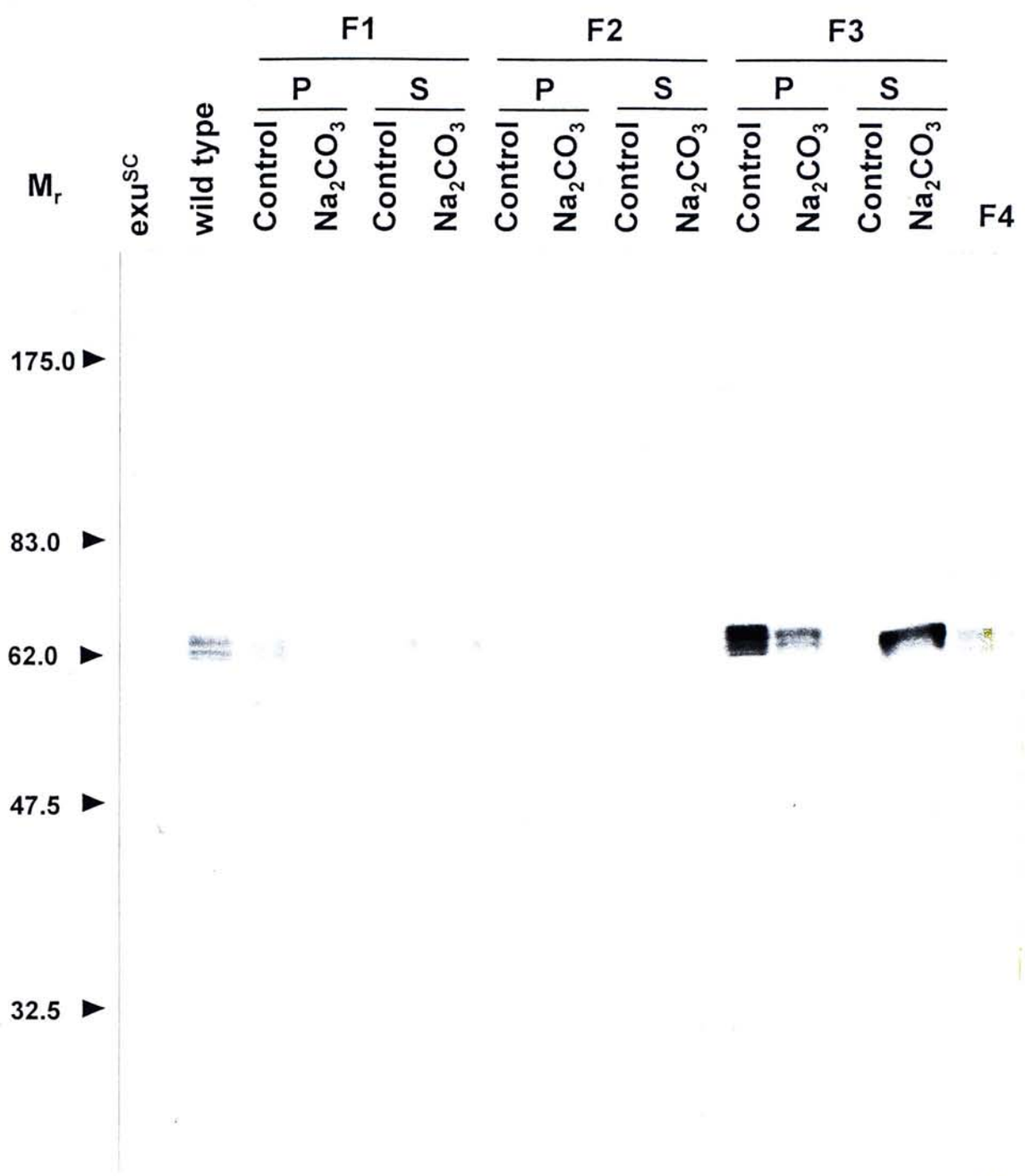


Figure 3.7 Subcellular fractionation of ovary extract.

Amount of exu protein was determined by western blot detecting with anti-exu antibody. Most of exu protein was associated with F3, the microsomal fraction. Sodium carbonate treatment released more than half of the exu protein from F3 while little was extracted in the control using PM buffer.

Lane: **F1**, subcellular fraction contained mainly large membranes and nuclei; **F2**, fraction contained lysosomes, mitochondria, and peroxisomes; **F3**, microsomal fraction; **F4**, the supernatant with little subcellular components; **P**, **S**, pellets and supernatants resulting from sodium carbonate treatment; **Na_2CO_3**, fraction extracted with sodium carbonate; **control**, fractions extracted with PM buffer as a control.

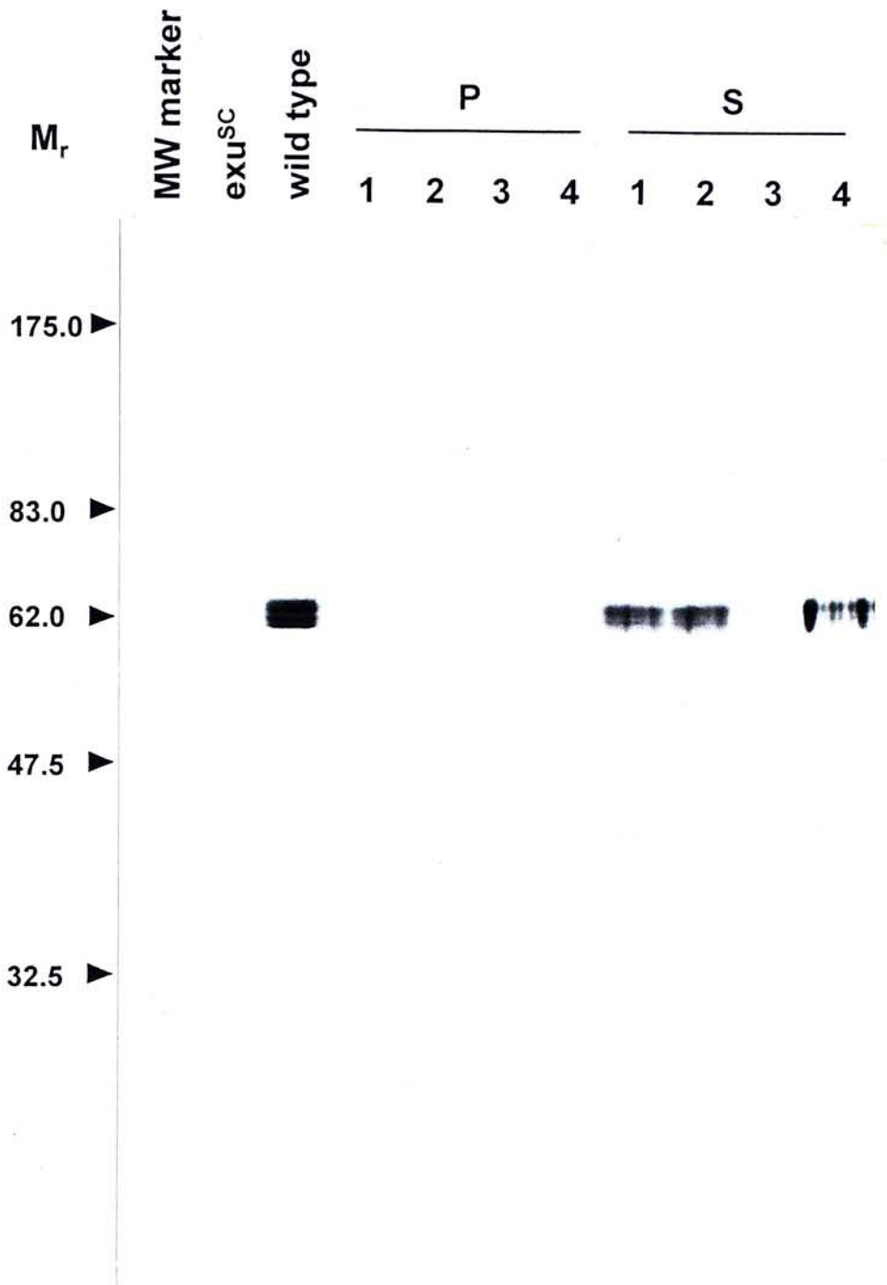


Figure 3.8 Analysis of exu protein and microtubules after cosedimentation using sodium carbonate released fraction.

After sodium carbonate treatment, microtubules were incubated with the extracts and were analysed after collection by centrifugation.

A. Western blot analysis of exu protein cosedimented with microtubules.

Lane: **exu^{sc}**, **wild type**, ovary extracts from *exu^{sc}* and the wild type respectively; **P**, **S**, pellets and supernatants resulting from incubations of; **1**, wild type ovary extracts, GTP, taxol and microtubules (MT); **2**, extracts, GTP, taxol, no MT; **3**, GTP, taxol, MT, no extracts; **4**, extracts and tubulin.

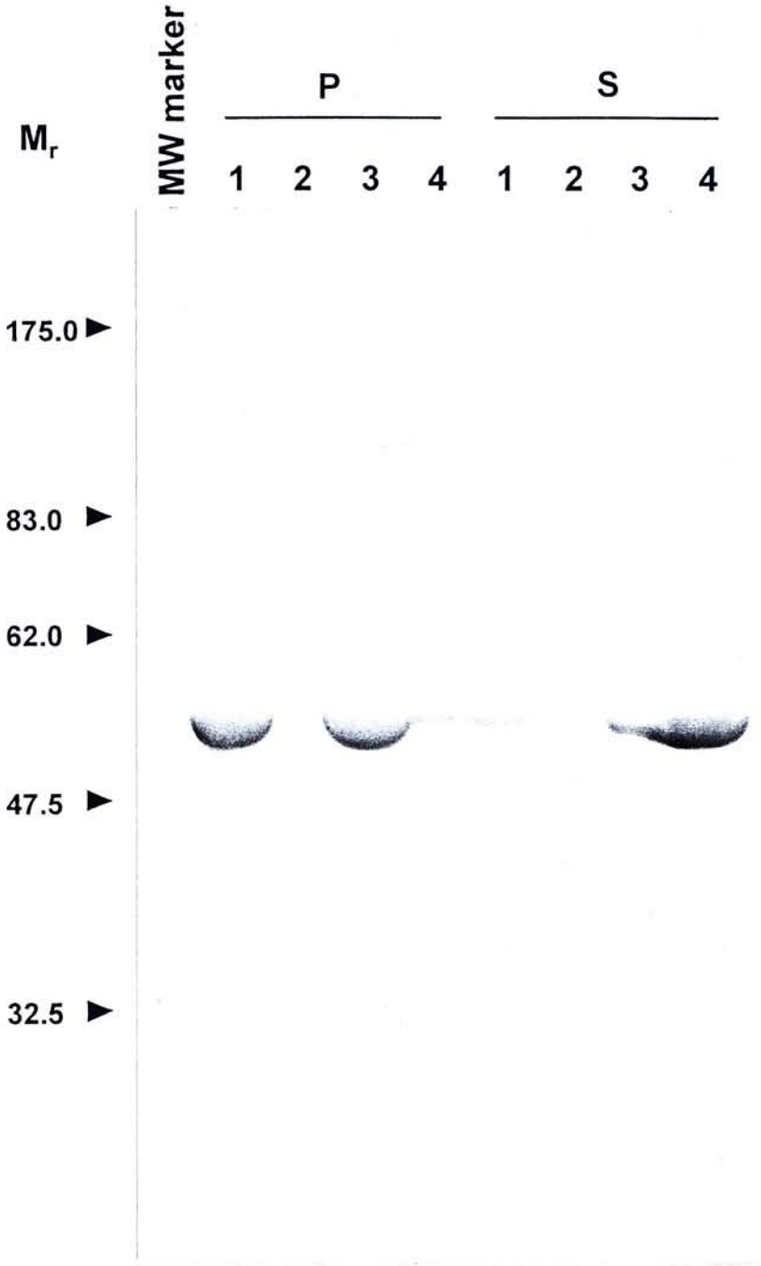


Figure 3.8 Analysis of exu protein and microtubules after cosedimentation using sodium carbonate released fraction.

B. Western blot analysis of tubulin. A duplicated blot of Figure 3.8A was detected by anti-β-tubulin antibody.

Lane: **P**, **S**; **1**, **2**, **3**, **4**, see Figure 3.8 A.

3.7 Cosedimentation of exu protein and microtubules in high ATP concentration

Conventional MAPs are selectively cosedimented with microtubules in the presence of high concentration of ATP, while both MAPs and motor proteins bind microtubules when ATP is depleted (Lee, 1989; Wiche, 1989; Rickard and Kreis, 1991; Hays et al., 1994; Arshad Desai, personal communication). By providing a high ATP concentration, microtubules were pelleted and analyzed for the presence of exu protein.

As exu protein was associated with the microsomal fraction, as described in the previous section, a more effective homogenization method was used. In addition, MAP-free phosphocellulose-purified tubulin was used as a source of microtubules.

3.7.1 Materials and Methods

3.7.1.1 Preparation of ovary extracts and microtubules sedimentation

The following protocol was essentially as described by Walczak et al. (1996) and Arshad Desai (personal communication) with modifications. About 200 pairs of flies' ovaries were dissected in PBS. The ovaries were briefly washed in PM buffer containing protease inhibitors (1.0 ug/ml Pepstatin A, 1.0 ug/ml Leupeptin, 1.0 ug/ml Aprotinin, 0.28 mM PMSF). The ovaries were dounce-homogenized at 4°C by 60 strokes using a tight-fit pestle. Further homogenization was done by sonication at 4°C with 15 times of 10 second-bursts. The homogenate was clarified by centrifugation at 78,000 r.p.m.(in a Beckman TLA-120.2 rotor) for 30 minutes at 4°C.

The supernatant was supplemented with a final concentrations of 2 mM ATP and 1 mM GTP on ice and then warmed to room temperature. Taxol was added to 5 mM and incubated for 5 minutes at 30°C. Additional taxol was added to a final concentration of 20 mM. Taxol stabilized microtubules were polymerized from phosphocellulose-purified bovine tubulin and were added to a final concentration of 0.2 mg/ml. After incubation for 30 minutes at 30°C, the sample

was layered onto a 40 % glycerol cushion containing 0.5 mM GTP, 10 uM taxol and protease inhibitors. Microtubules were sedimented at 42,000 r.p.m. for 40 minutes at 25°C in a Beckman TLS-55 swinging bucket rotor. The pellet was dissolved in 1X SDS sample buffer while the supernatant was concentrated by acetone precipitation and then dissolved in SDS sample buffer. The samples were analyzed by SDS PAGE and western blot analysis as described in sections 2.3.1.2 and 2.3.1.3.

3.7.1.2 Western blot using a chemiluminescent detection system

For higher sensitivity, a chemiluminescent detection kit, Western Star™ (Tropix) was used. Following electroblotting, the blot was washed briefly with PBS and then was blocked in blocking buffer (0.4 % I-block, 1X PBS, 0.1 % Tween 20) at 4°C overnight. The blot was either probed with the anti-exu antibody (dilution in 1:1500) or the anti-β-tubulin antibody (0.25 ug/ml) diluted in blocking buffer for 2 hours at room temperature. After washing three times for 15 minutes in wash buffer (PBS, 0.3 % Tween 20), the blot was incubated with alkaline phosphatase(AP)-conjugated secondary antibodies for 2 hours at room temperature. An AP-conjugated anti-rabbit antibody was used for detecting the anti-exu while an AP-conjugated anti-mouse antibody was used for probing the anti-β-tubulin. The blot was washed three times for 15 minutes in wash buffer and then twice for 5 minutes in assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM $MgCl_2$). After draining the excess assay buffer, the blot was flooded with 500 µl of substrate solution (5 µl CPD-star substrate, 25 µl Nitro-Block II, 470 µl assay buffer) and was then sandwiched in two sheets of clean transparencies. The blot was then exposed to X-ray film (Kodak X-OMAT™ AR).

3.7.2 Results

A more effective homogenization method was used for preparing the ovary extracts. The ovaries were homogenized with more extensive dounce-homogenization and sonication resulting in release of higher amount of exu protein.

As shown in Figure 3.9A, exu protein was found to cosediment with microtubules. A single band of exu protein was cosedimented with microtubules (lane P2) while no exu protein was sedimented in the absence of exogenous microtubules (lane P1). The wild type exu protein could be largely separated into four major bands or molecular weight isoforms on SDS PAGE. The band observed in lane P2 corresponded to the second band of exu protein when counting in an ascending order of relative molecular weight. It was interesting that only one of these major isoforms was cosedimented with microtubules whereas none of the other three isforms could be observed (lane P2). Therefore, the *in vitro* interaction between exu protein and microtubules was specific to only one of the isoforms.

The experiment was repeated using a chemiluminescent detection kit for western blot analysis. This assay provided higher sensitivity for detecting low level of protein. In the presence of both the wild type ovary extract and exogenous microtubules (lane P1, Figure 3.10A), exu protein was shown to cosediment with microtubules, while no exu protein was observed in the controls that contained ovary extracts only (lane P2), microtubules only (lane P3), and both ovary extracts and tubulin (lane P4). Again, only a single band was found to cosedimented with microtubules and this band corresponded to the second molecular weight isoforms of the multi-phosphorylated exu protein.

It was noteworthy that most of exu protein was not cosedimented with microtubules but remained unbound in the extracts. The amount of exu protein cosedimented with microtubules was relatively low in comparison with the total exu protein present in the ovary extracts. Several possibilities would account for this observation. The *in vivo* interactions between exu and microtubules might not have been reproduced well in an *in vitro* cosedimentation environment. Also, other components necessary for their interactions might not have been preserved. Therefore, a higher level of exu protein might have been associated with microtubules *in vivo*. Alternatively, it was possible that only a small proportion of

the total exu protein was needed for the interactions. Furthermore, as only the low molecular weight species interacted with microtubules, the hypo-phosphorylated form of exu protein was responsible for the interactions.

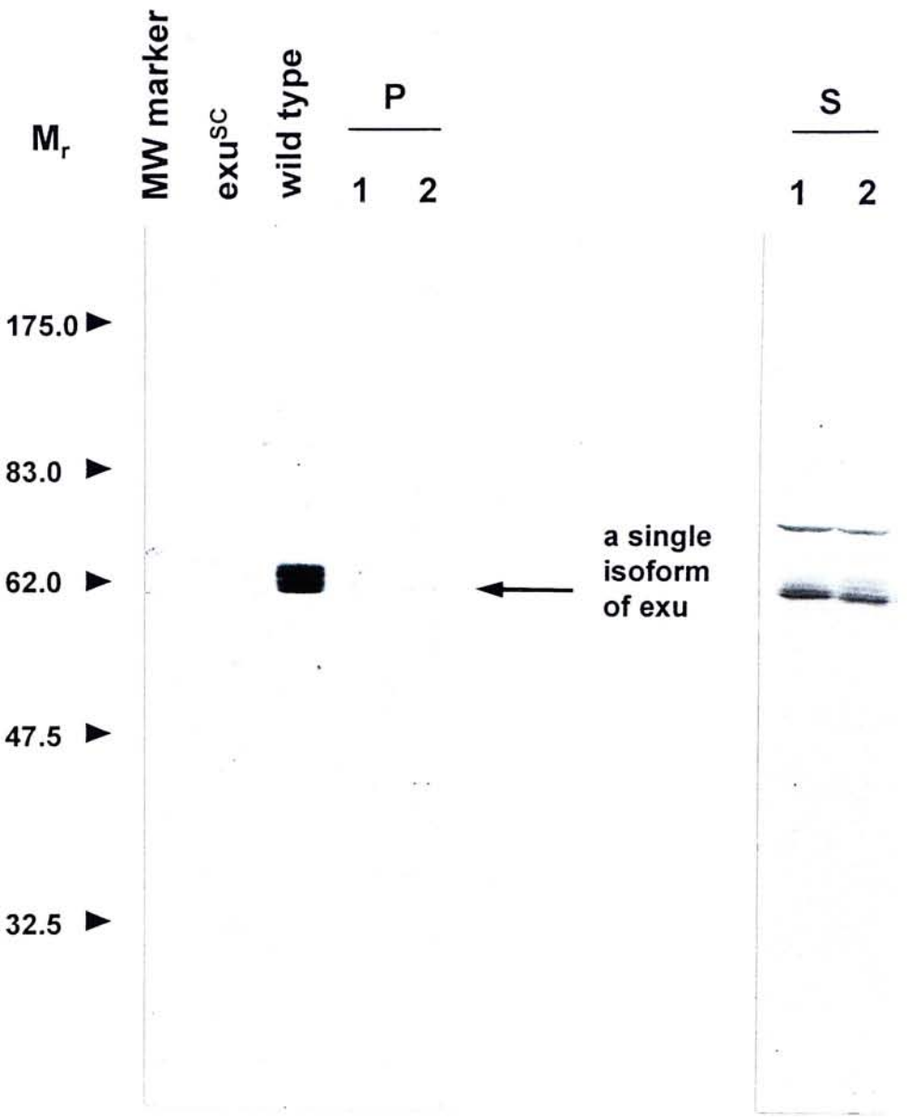


Figure 3.9 Cosedimentation of exu protein and microtubules in the presence of high ATP concentration.

A. Western blot analysis of exu protein cosedimented with microtubules.

Lane: **exu^{sc}**, **wild type**, ovary extracts from *exu^{sc}*and the wild type respectively; **P**, **S**, pellets and supernatants resulting from incubations of; **1**, wild type ovary extracts, GTP, and taxol; **2**, ovary extract, GTP, taxol and microtubules, in the presence of 2 mM ATP.

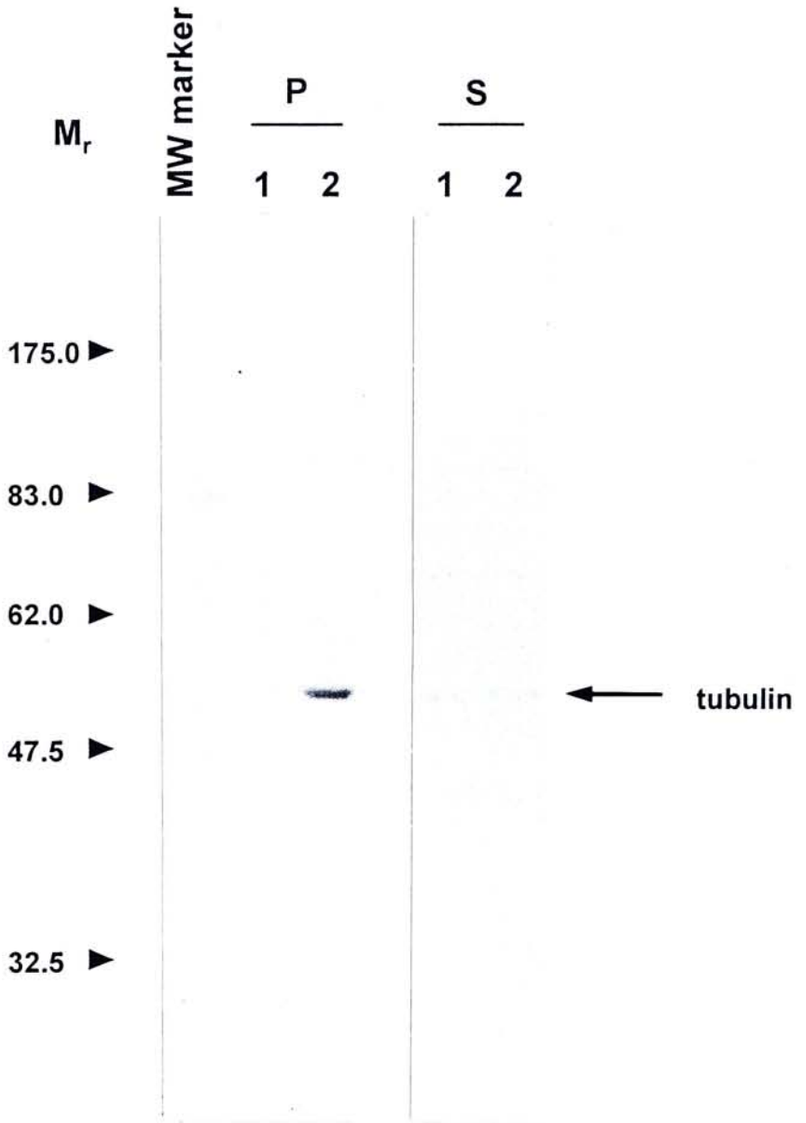


Figure 3.9 Cosedimentation of exu protein and microtubules in the presence of high ATP concentration.

B. Western blot analysis of tubulin. A duplicated blot of Figure 3.9A was detected by anti-β-tubulin antibody.

Lane: **P**, **S**, see Figure 3.9A.

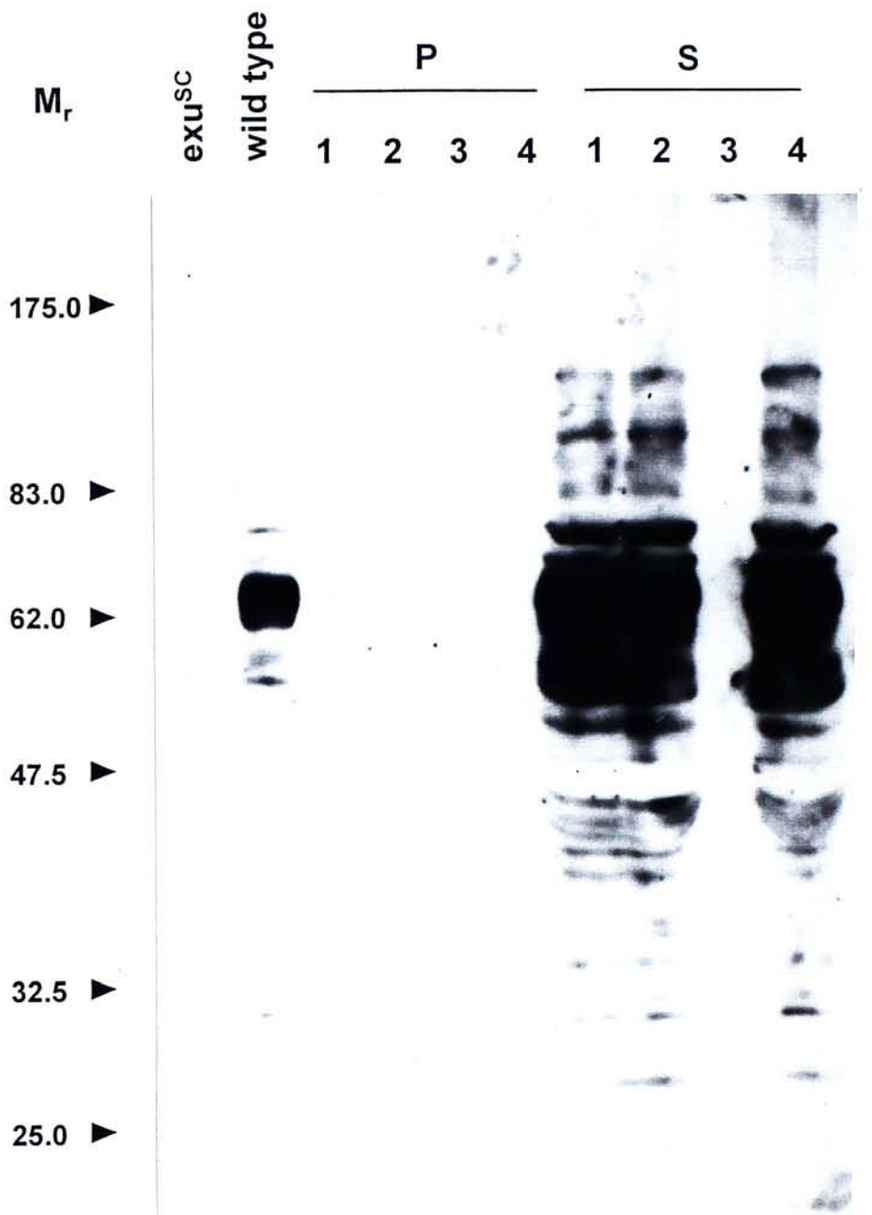


Figure 3.10 Dectection of cosedimentation of exu and microtubules in the presence of high ATP concentration by chemiluminescence blot

A. Western blot analysis of exu protein cosedimented with microtubules.

Lane: **exu^{sc}**, **wild type**, ovary extracts from *exu^{sc}* and the wild type respectively; **P**, **S**, pellets and supernatants resulting from incubations of; **1**, wild type ovary extracts, GTP, taxol and microtubules (MT); **2**, ovary extracts only, no MT; **3**, MT only, no ovary extracts; **4**, ovary extracts and tubulin, in the presence of 2 mM ATP.

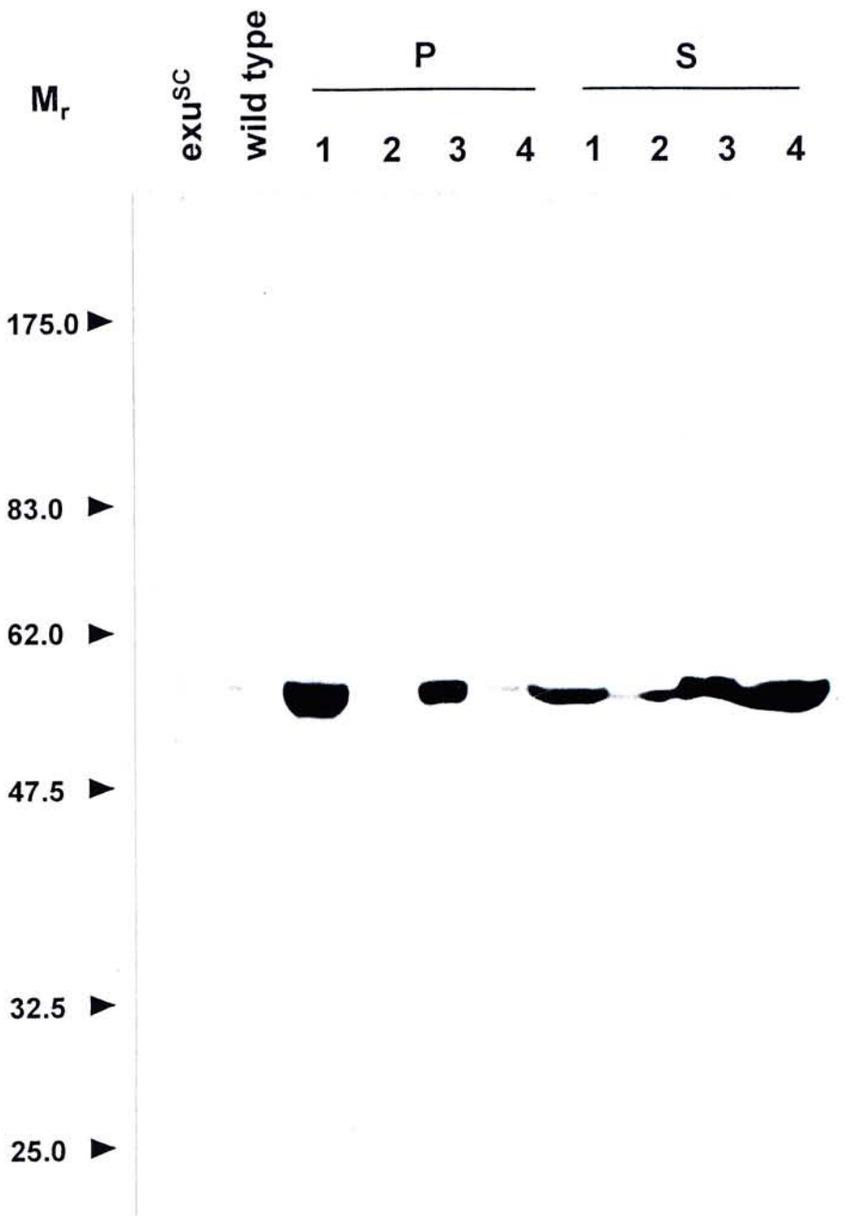


Figure 3.10 Dectection of cosedimentation of exu and microtubules in the presence of high ATP concentration by Chemiluminescence

B. Western blot analysis of tubulin. A duplicated blot of Figure 3.10A was detected by anti-β-tubulin antibody.

Lane: see Figure 3.10A.

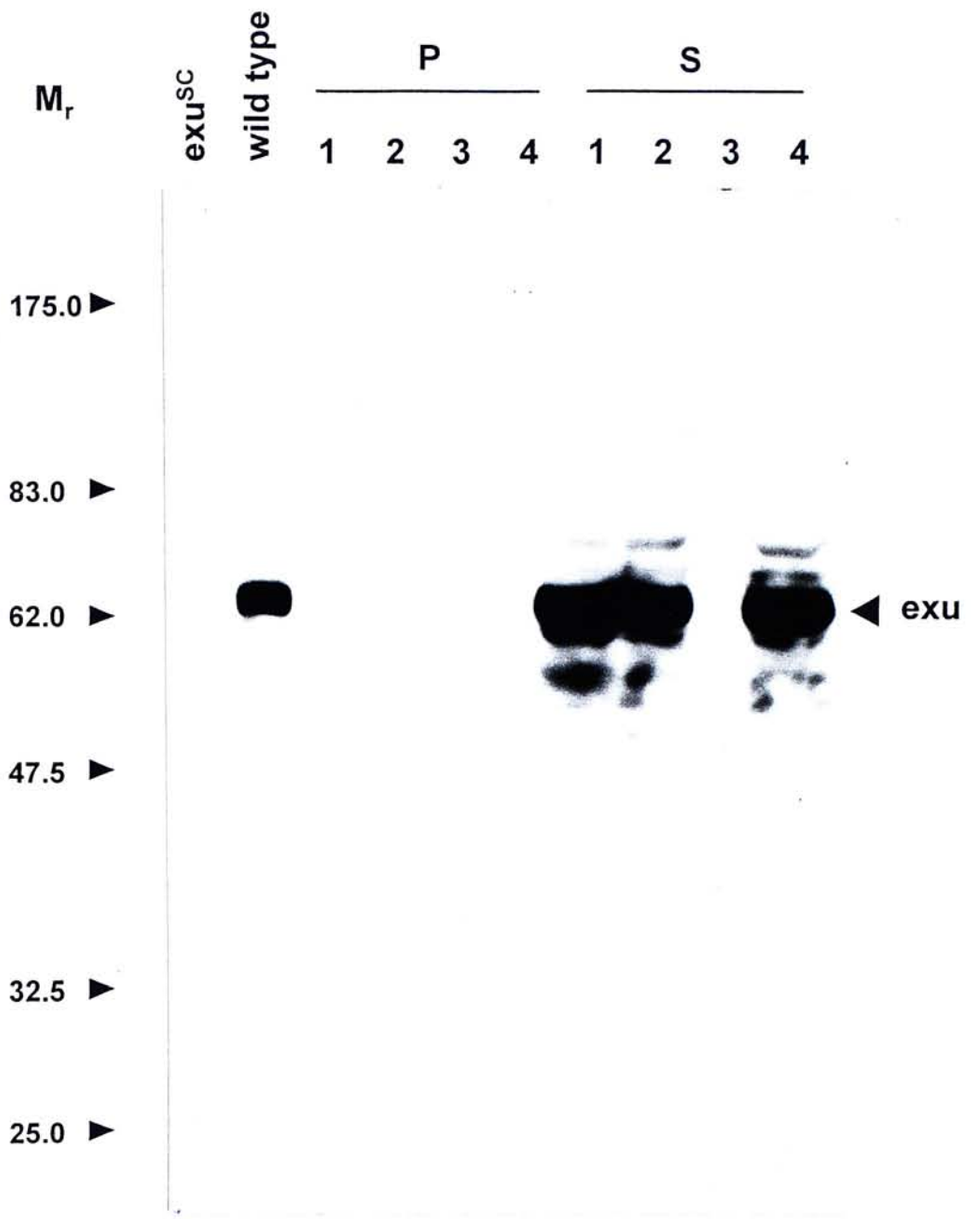


Figure 3.10 Dectection of cosedimentation of exu and microtubules in the presence of high ATP concentration by Chemiluminescence

C. Western blot analysis of exu protein cosedimented with microtubules. A dulpicated blot of Figure 3.10A was made by shorter exposure time which reduced the background. Exu protein is indicated by the arrow.

Lane: see Figure 3.10A.

3.8 Discussion

Localization of exu protein was found to be affected by the organization of microtubules. Using a microtubule destabilizing drug, colchicine, the localization of exu protein was completely abolished during oogenesis. Control injections with 1 % ethanol and with cytochalasin D confirmed that the localization was neither affected by the injection itself nor the stability of actin. Therefore, the localization of exu protein was sensitive to the organization of microtubules. When microtubules were destabilized, exu protein no longer localized in the oocytes from stages 1 through 9, indicating that exu protein requires an intact microtubule network for its localization and suggesting possible physical interactions between exu protein and microtubules.

Several approaches were used to determine whether exu protein interacted with microtubules. In the first approach, exu protein was purified by immunoprecipitation and then was allowed to bind microtubules. However, this method was unable to determine the interactions because microtubules seemed to bind to the protein G-agarose non-specifically.

Based on the observation of the dependence of localization of exu protein on microtubules, the cosedimentation approach that was used for isolation of MAPs was adopted. Various conditions for MAPs cosedimentation were used but a major difficulty was encountered in which only a low concentration of exu protein was extracted in the ovary homogenate. The usual homogenization methods employed by other MAPs cosedimentation experiments seemed to be insufficient to extract exu protein as most of it was lost after clarification by centrifugation. Thus, the limitation of the amount of exu would in fact account for the low amount of exu protein cosedimented with microtubules in sections 3.4 and 3.5.

By subcellular fractionation, it was found that the low concentration of exu in the ovary extracts was due to the association of exu to the microsomal fraction, which was lost during the clarification step. To release exu protein from the microsomal fraction, a more effective homogenization and sonication procedure was employed. Fortunately, more exu protein was extracted into the soluble fraction (section 3.7). About half of the total exu protein could be extracted and the

cosedimentation experiment was repeated with these extracts. Besides, phosphocellulose-purified bovine tubulin was used instead of rat tubulin as the former was the one usually used in MAPs sedimentation experiments.

The experimental results showed that exu did cosediment with microtubules. Although only a small proportion of total exu protein bound microtubules, their *in vitro* association was specialized to only one molecular weight isoform which was hypo-phosphorylated. Thus the interaction between exu protein and microtubules appeared to be very specific.

The experimental results shown here extended the model proposed by Lane and Kalderon (1994), in which exu protein may play a role similar to that of MAPs. At the beginning of mid-oogenesis (stages 6-7), the posteriorly located MTOC degenerates and the new microtubules nucleating region establishes at the anterior of the oocyte providing a uniform gradient necessary for the correct localization of maternal determinants (Theurkauf, et al. 1992, 1993). The degeneration of the posterior MTOC has been shown to depend on a germline protein kinase A (PKA) as mutations of PKA resulted in the persistence of the posterior MTOC at mid-oogenesis (Lane and Kalderon, 1994). It was proposed that the germline PKA is activated by signals generated from the posterior polar follicle cells which in turn destabilizes the posterior MTOC (Lane and Kalderon, 1994). A MAP2-like protein was thought to bridge between PKA and microtubules MTOC (Lane and Kalderon, 1994). MAP2 has been shown to be phosphorylated by PKA in vertebrate brain extract (Sloboda et al., 1975; Theurkauf and Vallee, 1982). Furthermore, it can stabilize microtubules by their associations, and this stabilizing effect can be released by PKA phosphorylation of MAP2 (Jameson and Caplow, 1981). The posterior MTOC is likely to be stabilized by binding of the MAP2-like protein at the early oocytes (stages 2-6). At the beginning of mid-oogenesis (stage 6), the MAP2-like protein may be phosphorylated by PKA and dissociated from microtubules which lose the stabilization and thus the posterior MTOC degenerates.

As the experimental results shown here suggested that exu was a MAP, it might function to regulate the stability of microtubules. It has been shown that exu protein was localized exclusively in the oocyte at very early stages of oogenesis, and this localization might facilitate its phosphorylation by PKA and its interactions with microtubules within the oocyte. As only a small amount of exu protein interacted

with microtubules, exu protein could not be associated with the whole population of microtubules but rather only with particular structures such as the nucleating centers of microtubules or MTOC. MTOC is composed of a ring shaped complexes containing γ-tubulin as well as several other proteins and plays a central role in regulating microtubule assembly (Moritz et al., 1995; Zheng et al., 1995; reviewed by Oakley, 1995). Thus exu protein may take part in the ring shaped complex to stabilize the MTOC.

Besides, exu is a phosphoprotein which exists in multi-phosphorylated isoforms and exu from the ovary extracts could be phosphorylated by several specific protein kinases, including PKA (Cheung et al., unpublished data, manuscript in preparation). The specific interaction between one of the hypo-phosphorylated isoforms of exu protein and microtubules suggested that exu might be involved in the regulation of stability of MTOC by its phosphorylation.

Although exu protein is shown here to interact with microtubules, it would be interesting to determine if there are interactions between exu and MTOC. Further studies may involve a cytological and genetic approach to analyze the distribution of MTOC within egg chambers of *exu* mutants. An abnormal distribution of MTOC will be observed in the *exu* mutants if exu protein is involved in regulating the stability of MTOC. In addition, analysis of distribution of both exu protein and MTOC within egg chambers may reveal their *in vivo* interactions. Immunohistochemical staining can be used to analyze the distribution of exu protein and γ-tubulin, which is one of the components of MTOC. If exu protein interacts with MTOC, they will be expected to colocalize within the egg chambers. Furthermore, egg chambers of the PKA mutants, which have abnormal distribution of MTOC at both ends of the oocyte, can be examined in parallel with those of the wild type to determine if exu protein is always colocalized with MTOC. Another studies may involve a biochemical approach to analyze the interactions between exu and MTOC. MTOC can be purified according to the protocol described by Zheng et al. (1995) and the interactions with exu protein can then be analyzed.

Chapter 4

Future prospects

Although the deletion analysis did not identify the functional domains of exu protein, it had restricted the potential functional regions by determining the dispensability of 62 amino acid residues at the C-terminus.

Luk et al. (1994) has demonstrated the existence of conservation in the exu coding sequence among different *Drosophila* species. There are several conserved regions found within the coding sequences of the three evolutionary diverged species, *D. melanogaster*, *D. virilis* and *D. pseudoobscura*. Two highly conserved regions, one locating near the N-terminus with greater than 90 % of homology and one locating in the middle of the coding sequence with almost 100 % homology, were observed among the three species. Other three regions with intermediate sequence conservation were also found and PEST domains were located within one of these regions.

Functionally important domains of a protein usually have high structural restraints and they are conserved, at least at the amino acid sequence level, among homologues of different species. Thus functional domains are expected to reside within the conserved regions of the exu protein coding sequence. Deletion analysis within the conserved regions will presumably locate the functional domains of exu.

As exu protein has been proven to bind microtubules *in vitro*, it is now possible to delineate the interacting domain(s) of exu protein. Deletion analysis will be performed to analyze the potentially important regions on the coding sequence. Taking advantage of the existing deletion mutants, mutant exu proteins prepared from each mutant can be used for cosedimentation assay to further identify the potential microtubule interacting domain(s) of exu protein.

Appendix A

Supplementary protocols

1. SDS PAGE

Part I Gel setup

The gel unit of thickness 0.8 mm was assembled and sealed with tape at the bottom. A 10 % gel mix was prepared according to Table A1. A gel plug was made by mixing 2 ml of the above gel mix with 16.5 µl 20 % ammonium persulphate and 6.5 µl TEMED. This gel mix was thoroughly mixed and pipetted into the gel set. The gel plug was quickly polymerized and set within 5 minutes. After adding 33.5 µl 20 % ammonium persulphate and 13.5 µl TEMED, the remaining 10 % gel mix was thoroughly mixed and pipetted into the gel set. A thin layer of water was overlaid on the gel to avoid direct contact with air. The gel was then allowed to polymerize overnight.

A stacking gel mix was prepared according to Table A2. The stacking gel mix was mixed with 35 µl 20 % ammonium phosphate and 15 µl TEMED and pipetted into the gel set. A comb was inserted and the gel was polymerized for 45 minutes.

Part II Electrophoresis

Protein samples were loaded into each wells and equal amounts of 1X SB were loaded into the empty wells. Electrophoresis was run under a constant current of 25 mA for about 4 hours.

Table A1 Composition of separating gel

	5 %	8 %	10 %	12.5 %	15 %	20 %
Tris-HCl, 1.5 M (pH 8.8)	4.99 ml	4.99 ml	4.99 ml	4.99 ml	4.99 ml	4.99 ml
Acrylamide, 30 %	3.34 ml	5.34 ml	6.67 ml	8.34 ml	10.00 ml	13.34 ml
SDS, 20 %	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
water, double-distilled	11.57 ml	9.57 ml	8.24 ml	6.57 ml	4.91 ml	1.57 ml

Table A2 Composition of stacking gel

	5 %
Tris-HCl, 0.5 M (pH 6.8)	2.5 ml
Acrylamide, 30 %	1.7 ml
SDS, 20 %	0.1 ml
water, double-distilled	5.7 ml

2. Western blot analysis

The gel together with a nitrocellulose paper (Schleicher & Schuell) pre-wetted with water, were soaked in transfer buffer (2.91 g Tris, 1.47 g glycine, 1.9 ml 10 % SDS, 100 ml methanol, make up to 500 ml with double distilled water) for 15 to 30 minutes. The gel and the nitrocellulose were sandwiched in Whatman 3MM paper, that have been presoaked with transfer buffer. This sandwich was placed on a semi-dry blot apparatus (Owl) and excess buffer was removed. The proteins of the SDS gel were transferred electrophoretically under a constant voltage of 15 V for 45 minutes.

At the end of transfer, the nitrocellulose blot was briefly washed in double-distilled water and then blocked in 5 % (w/v) skimmed milk in TTBS (10 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 0.05 % Tween 20) for 1 hour at room temperature. After blocking, the skimmed milk solution was replaced by a primary antibody diluted in TTBS with 1 % BSA. For the detection of exu protein, a rabbit anti-exu antibody was used in a dilution of 1 : 1000. After an overnight incubation at 4°C, the blot was washed three times for 15 minutes in TTBS. The blot was incubated with a goat anti-rabbit antibody conjugated with alkaline phosphatase, used at a dilution of 1 : 5000 in TTBS with 1% BSA, for 2 hours at room temperature. The blot was washed three times for 10 minutes and then developed with NBT (0.033 % w/v) and BCIP (0.017 % w/v) in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). The reaction was stopped by rinsing the blot with water.

3. Preparation of DNA template for DIG-labeling

The plasmid (p1122) containing the *bcd* cDNA clone was digested with EcoRI and EcoRV to release a 1.7 kb fragment for the synthesis of a DNA probe. The following components were mixed in a microfuge tube and incubated overnight at 37°C:

(1) double distilled water	12.6 µl
(2) NEB buffer for EcoRI	2.0 µl
(3) BSA (10X, NEB)	2.0 µl
(4) 2 µg DNA (p1122)	2.4 µl
(5) EcoRI (20 U/µl, NEB)	0.5 µl
(6) EcoRV (20 U/µl, NEB)	0.5 µl

The digested DNA was mixed with 2 µl of 6X gel-loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % glycerol in water, Sambrook et. al., 1989) and separated by electrophoresis on a 1 % agarose gel containing 0.5 ug/ml of ethidium bromide for one hour (or until the dye front reached the end of gel) at a constant voltage of 100V. Using a hand-held UV lamp (long wavelength), the 1.7 kb-EcoRI/ RV fragment was located and excised. The gel piece was placed in a dialysis tubing and the DNA was recovered by electroelution (Sambrook et. al., 1989).

The eluted DNA was purified through phenol/ choroform extraction and then precipitated by ethanol precipitation (Shapiro, 1981). One-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of ice-cold absolute ethanol were added. The DNA was precipitated at -20°C for one hour. The precipitated DNA was collected by centrifugation at 10,000 x g for 15 minutes. The pellet was washed with 70 % ethanol and once again collected by centrifugation. The DNA pellet was air-dried for 5 minutes and then resuspended in 10 µl of double-distilled water. This DNA would be used as the template for DIG-labeling.

4. Test of labeling efficiency of DIG-labeled DNA probe

The efficiency of the labeling reaction was tested by immobilizing the probe on nitrocellulose paper, detecting with an anti-DIG antibody and NBT / X-phosphate color reaction as described below.

A small amount of probe (1 μl) was boiled in 5 μl 5X SSC (20X stock: 3 M NaCl, 0.3 M sodium citrate, adjust pH to 7.0 with 10 N NaOH) for 5 minutes. In addition, a DIG-labeled DNA and an unlabelled DNA from the kit were also boiled to serve as the positive control and negative control respectively They were then quickly chilled on ice and 1 μl of the content was spotted onto a small strip of nitrocellulose paper. The spots were air-dried and cross-linked by UV using a UV Stratalinker 1800. The nitrocellulose strip was wetted with 2X SSC and washed twice in PBT (PBS, 0.1 % BSA, 0.2 % Triton X-100). It was then incubated with an alkaline-phosphatase conjugated anti-DIG antibody (Boehringer) in PBT for 30 minutes. After washing twice in AP buffer, the reaction was developed with NBT (0.45 % v/v) and X-phosphate (0.3 % v/v) in AP buffer with 0.1 % Tween 20. The reaction was stopped by washing several times in PBS. The efficiency of the labeling reaction was determined by the intensity of the color spot produced by the probe as compared to the labeled control.

Appendix B

Reagents

ATP (0.1 M)

0.1 M ATP was made by dissolving 60 mg of ATP (Sigma, disodium salt) in 0.8 ml double-distilled water and the pH was adjusted to 7.0 with 1 N NaOH. The volume was adjusted to 1 ml and stored in aliquots at -70°C.

BCIP

0.5 g of BCIP (Sigma) was dissolved in 10 ml of 100 % dimethylformamide and the solution was stored at -20°C.

DTT (1 M)

3.09 g of DTT (Pharmacia)was dissolved in 20 ml of 0.01 M sodium acetate (pH 5.2) and the solution was stored at -20°C.

EDTA (0.5 M)

18.61 g EDTA (BDH, disodium salt)was dissolved in 80 ml double-distilled water and the pH was adjusted to 8.0 with NaOH. The resulting solution was adjusted to 100 ml and sterilized by autoclaving.

EGTA (100 mM)

3.8 g of EGTA (Sigma) was dissolved in 80 ml double-distilled water and the pH was adjusted to 11 with NaOH. After dissolving all EGTA, the pH was adjusted to 8.0 and the solution at 4°C for use for one month.

Electrode buffer (10X)

30.3 g of Tris base (USB) and 142.7 g of glycine (USB) were dissolved in 1000 ml double-distilled water.

GMM

25 ml of Canada Balsam (Sigma) was mixed with 1.7 ml methyl salicylate (Sigma) with agitation overnight.

GTP (0.1 M)

0.1 M GTP was made by dissolving 61 mg of GTP (3 moles of Na and 1 mole of H_2O per mole of GTP) (Sigma, Type III) in 0.8 ml double-distilled water and the pH was adjusted to 7.0 with 1 N NaOH. The volume was adjusted to 1 ml and stored in aliquots at -70°C.

Heparin

Heparin (Sigma, sodium salt) was dissolved in 4X SSC at a concentration of 50 mg/ml and stored at 4°C.

NBT

0.5 g of NBT (Sigma) was dissolved in 10 ml of 70 % dimethylformamide and the solution was stored at -20°C.

4 % paraformaldehyde

0.4 g of paraformaldehyde (Fluka Chemica) was dissolved in 10 ml PBS and the solubility was increased by addition of 10 µl 10 N NaOH and heating to about 60°C. The solution was neutralized with 20 µl of 5 N HCl and after cooling to room temperature.

PIPES (1.0 M)

30.24 g of PIPES (FW 302.36) (BDH) was dissolved in 80 ml of double-distilled water and the pH was adjusted to 6.8 with NaOH. (PIPES would gradually dissolved as pH increased.) The solution was adjusted to 100 ml.

Proteinase inhibitors

aprotinin (Sigma) - stock solution of 10 mg/ml was made by dissolving in 0.01 M HEPES (pH 8.0) and stored at 4°C.

leupeptin (Sigma)- stock solution of 10 mg/ml was made by dissolving double-distilled water and stored at 4°C.

pepstatin A (Sigma)- stock solution of 1 mg/ml was made by dissolving in methanol and stored at 4°C.

PMSF (Sigma)- Stock solution of 200 mM was made by dissolving in ethanol and stored at 4°C.

Sonicated salmon sperm DNA

Salmon sperm DNA (Sigma, sodium salt)was dissolved in water at a concentration of 10 mg/ml. The concentration of NaCl was adjusted to 0.1 M and the solution was extracted with phenol chloroform. The DNA solution was sonicated and precipitated by adding 2 volumes of ice-cold ethanol. The DNA was dissolved in double-distilled water and the exact concentration was determined by OD_{260}.

SSC (20X)

175.3 g of NaCl and 88.2 g of sodium citrate were dissolved in 800 ml double-distilled water and the pH was adjusted to 7.0 with NaOH. The final volume was adjusted to 1000 ml and the solution was sterilized by autoclaving.

Transfer buffer

2.91 g Tris base, 1.47 g glycine, 1.9 ml 10 % SDS and 100 ml methanol were mixed and adjusted to 500 ml with double-distilled water.

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